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Final Report on the Evaluation of Four Toxic Chemicals in an 'In vivo/In vitro' Toxicological Screen: Acrylamide, Chlordecone, Cyclophosphamide, and Diethylstilbestrol

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

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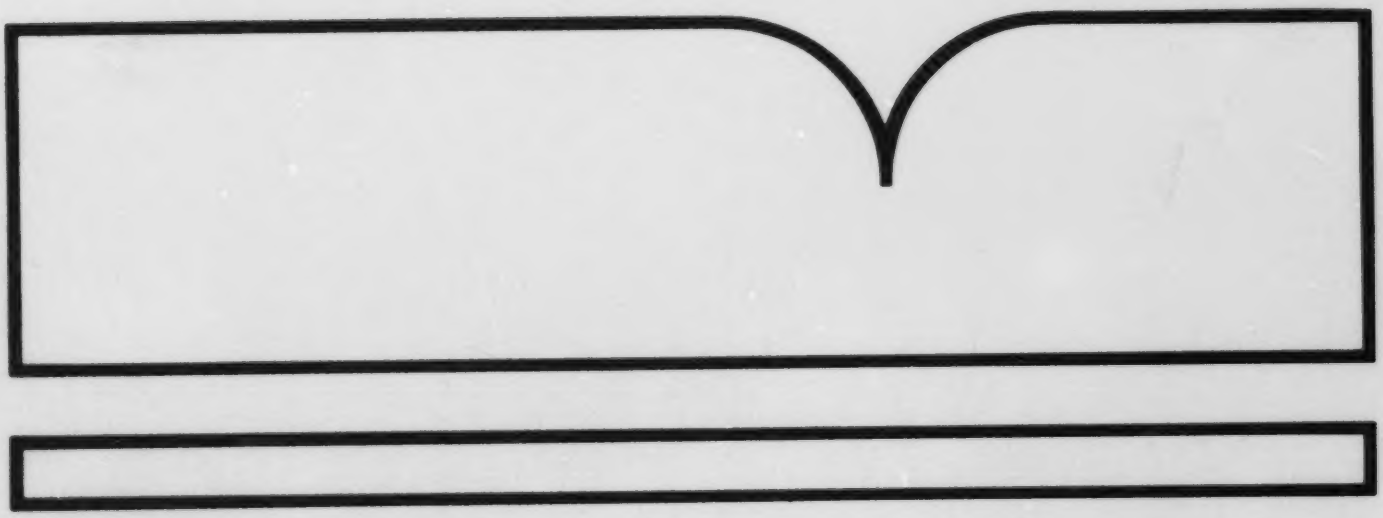

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Final Report on the Evaluation of Four Toxic Chemicals in an *In Vivo*/*In Vitro* Toxicological Screen:

Acrylamide, Chlordecone, Cyclophosphamide, and Diethylstilbestrol

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16. ABSTRACT An in vivo/in vitro Toxicological Screen (Tox Screen) has been developed for screening large numbers of wastes for biological activity. Emphasis is placed on identifying a wide range of potential toxic responses by employing diverse test methods with toxic endpoints in mutagenesis/carcinogenesis, general toxicology, neurotoxicology, reproductive toxicology, teratology, and immunotoxicology. Oral administration of waste material is given to rats for 10 consecutive days after which the whole animal, body tissues and fluids are evaluated for toxicity. The Tox Screen is being validated to ensure that the protocol will be capable of detecting biological activity and to identify those assays which most readily detect toxicity. The most accurate and sensitive assays would be used as a Prescreen for the entire protocol. Results of the validation study with 4 toxic chemicals are included. Acrylamide produced its greatest effects in the neurotox assays and in mutagenesis. The reproductive assays were most sensitive in detecting the toxicity of chlordecone. The toxicity of cyclophosphamide was most readily identified by the immunological and mutagenesis tests. Diethylstilbestrol produced dose-response effects in all disciplines. The Tox Screen is currently being re-evaluated to answer questions concerning the length of exposure and the need to add and/or eliminate certain tests to increase sensitivity and accuracy. In addition, several new areas of research are being persued in additivity/synergism/antagonism, chronicity and in vitro to in vivo extrapolation.

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Foreword

The many benefits of our modern, developing industrial society are accompanied by certain hazards. Careful assessment of the risk of existing and new man-made environmental hazards is necessary to establish sound regulatory policy. Environmental regulations enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our nation's population.

The Health Effects Research Laboratory conducts a coordinated environmental health research program in toxicology and clinical studies. These studies address problems in air pollution, radiofrequency radiation, environmental carcinogenesis, and the toxicology of pesticides, as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for radiofrequency radiation guidelines. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator.

The intent of this document is to outline the development of a cost- and time-effective test method in toxicology to identify biologically active hazardous waste samples and process residuals. The development of test methods for identifying biologically active wastes provides necessary information to aid EPA in setting regulatory standards for the management of hazardous wastes.

F.G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

Executive Summary

An *in vivo*/*in vitro* Toxicological Screen (Tox Screen) has been developed to use methods that are designed to be rapid, inexpensive and capable of screening large numbers of wastes for biological activity. Emphasis is placed on identifying a wide range of potential toxic responses associated with each waste by employing diverse test methods. The toxic endpoints identified by the screen include mutagenesis/carcinogenesis, general toxicology, neurotoxicology, reproductive toxicology, teratology, and immunotoxicology. The protocol, called the Toxicological Screen, involves the oral administration of waste material to rats for 10 consecutive days. At the end of the 10-day period the whole animal, body tissues and fluids are evaluated for toxicity. A major goal of the Toxicological Screen is to maximize the amount and type of potential health effects information that can be obtained by exposing metabolically competent intact animals to complex waste mixtures.

The Toxicological Screen is being validated by using a series of compounds of known and defined toxicity. The validation study ensures that the protocol will be capable of detecting biological activity associated with each compound and identifing those assays which most readily detect the toxic potential of the compounds. A selected number of the most accurate and sensitive assays could be used to constitute a Prescreen for the entire protocol. The Prescreen could then be used (with an abbreviated form of the *in vivo* exposure regime) as a rapid means of prioritizing waste samples to be evaluated in the full Tox Screen. It is important to note here that a large number of compounds of known toxicity and no toxicity must be evaluated in the Tox Screen before bioassay tests for the Prescreen would be initiated on wastes. These tests would constitute a validation study to establish the sensitivity, specificity and accuracy of the Tox Screen.

Initial trials of the validation study with acrylamide, chlordecone (kepone), cyclophoshamide, and diethylstilbestrol (DES) demonstrate the feasibility of the Toxicological Screen for detecting biological activity of chemicals.

Acrylamide, evaluated at five test concentrations ranging from 3.75 to 60 mg/kg in rats, produced its greatest toxic effects in motor activity, responses to acoustic stimuli (neurotoxicology), and in the induction of sister chromatid exchanges (mutagenesis). Most other tests resulted in positive responses at the highest concentration tested, a concentration which produced 30% lethality.

The reproductive assays were most sensitive in detecting the toxic potential of chlordecone (evaluated at 0.625 to 10.0 mg/kg in rats). Testicular sperm was markedly reduced, demonstrating that chlordecone is a reproductive toxin. This observation agrees with previously reported studies of sterility in chemical workers employed in chlordecone manufacturing in Hopewell, Virginia. Had this sample been an unknown hazardous waste mixture the observed results would suggest the presence of a potential reproductive toxin and would indicate a need for further research.

The toxicity of cyclophosphamide (1.5 to 24 mg/kg in rats) was most readily identified by the immunological and mutagenesis tests. Most other tests responded at the highest concentration tested, which resulted in 70% lethalities. Cyclophosphamide produced mutagenic metabolites in the urine of treated rats. These metabolites were detectable using the Salmonella histidine reversion assay. Increases in sister chromatid exchanges were identified in the bone marrow at each exposure concentration (1.5 to 24.0 mg/kg rat). Dose-response relationships were established for each of the immunotoxicity tests with cyclophosphamide. Also, there were significant reductions in thymus and spleen weights at all concentrations tested. Body weight reductions were observed only at the two highest doses. These results indicate that generalized body weight loss is not predicitive of potential immunotoxicity.

The exposure of animals to DES also resulted in the generation of a number of dose-response relationships in several areas. Almost all parameters evaluated in general toxicology, neurotoxicology, immunotoxicology, reproductive toxicology, and teratology were affected at the lowest concentration tested, 31.25 mg/kg. The only test category which did not detect the toxicity of DES was mutagenesis which is not surprising since this toxin is not a bacterial mutagen nor does it induce SCEs.

The results of the validation study to date suggest that the Tox Screen is capable of detecting a range of biological activity of the pure compounds as well as the type of activity associated with the pure compounds. Four compounds, however, are insufficient for establishing the required sensitivity (0.9), specificity (0.75), and accuracy specified by OSW or to to select the best tests for the abbreviated prescreen. The number of compounds required to identify prescreen assays and to establish the accuracy of the Tox Screen (with a 90% sensitivity) requires a minimum of 15 to 20 biologically active compounds for each of the six disciplines of the Tox Screen (assuming no overlap in toxicity) and 15 to 20 control compounds without biological activity (assuming 100% overlap in toxicity across endpoints). This results in a total of 105 to 140 compounds. The evaluation of this many compounds may be a prohibitive task because of both time and cost limitations.

As a result the Tox Screen is being re-evaluated with respect to the treatment protocol and the toxicological assays which it employs. Several questions must be answered as part of this re-evaluation process and before validation of the Tox Screen can be designed.

1. Is a 10 day exposure of a waste material adequate for predicting chronic effects? Should a 30 or 90 day exposure regime be employed?

2. Are there toxicity tests which could be added or deleted to improve the overall performance and sensitivity of the Tox Screen?

3. Can certain in vivo tests be replaced with in vitro tests to reduce cost without reducing sensitivity or accuracy?

4. Is 90% sensitivity and 75% accuracy actually required?

In addition to addressing the above questions, several new areas of research are being persued as part of an ongoing developmental program in the evaluation of complex mixtures.

1. The development of short term *in vitro* genetic mutation and liver toxicity bioassays for the identification of hazardous waste samples possessing acute and chronic biological effects.

2. The development of new and improved *in vivo*/*in vitro* screening methods in the areas of liver toxicology, neurotoxicology, immunotoxicology, and developmental biology for addition to the integrated Tox Screen.

3. The investigation and resolution of some of the basic toxicological problems related to complex mixtures and the screening assay (i.e., acute vs. chronic effects and comparison of in vivo and in vitro methodologies).

This report covers a period from April 8, 1983 to June 22, 1985 and work was completed as of April 21, 1986.

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SECTION 1

INTRODUCTION

The disposal of the vast quantity of wastes generated in the United States each year is of great economic, public and environmental concern. The development of suitable waste management methods requires a means of identifying the "hazardous" proportion of the nation's total wasteload. It is only with the identification of hazardous wastes that proper management procedures (e.g. storage, transportation and disposal methods) can be utilized to ensure public health and safety and integrity of the environment.

Over the past 10 years much research has been directed toward the identification and hazard assessment of a variety of complex mixtures and environmental pollutants[1]. Many programs have relied on a totally *in vitro* battery of tests of increasing complexity to identify complex mixtures with the potential for chronic toxicity (eg. mutation, cancer)[2]. Others have estimated the hazard associated with pollutants by identifying the chemical components of a complex mixture. This information is then used in combination with computer programs designed to estimate the biological activity of the mixture. Athough both approaches provide valuable information concerning sample activity, they do not estimate the potential synergistic or antagonistic effects a pollutant may exhibit in the intact animal. An *in vivo* or combined *in vivo*/*in vitro* toxicological screen may provide the best first approximation of the toxicity of a complex mixture.

The Health Effects Research Laboratory (HERL) at Research Triangle Park, NC has developed an *in vivo*/*in vitro* toxicological screen for the identification of biologically active hazardous waste mixtures. The screen involves the short-term repeated exposures of rodents to waste materials followed by toxicological assessment of the whole animal, body tissues and fluids. Emphasis is placed on identifying several types of biological activity associated with wastes in addition to the degree of activity.

The protocol, called the Toxicological Screen (Tox Screen), has been developed to provide the informational bases required for aiding regulatory judgments regarding the classification of wastes under the Resource Conservation and Recovery Act (RCRA). Any waste classified (or listed) as hazardous would be subject to stringent disposal methods as specified under Subtitle C of RCRA.

The Tox Screen has been reviewed by an external committee of scientists[3]. Their recommendations and comments have been incorporated into the Tox Screen's design. Additional review will be made once the final design of the Screen has been established.

This report reviews the design and results of the four toxic chemicals (acrylamide, chlordecone, cyclophosphamide, and diethylstilbestrol) tested in the Tox Screen, current limitations of the screen, potential solutions to these limitations and research plans.

SECTION 2

CHEMICAL SELECTION AND EVALUATION

Acrylamide, chlordecone, cyclophosphamide, and diethylstilbestrol were selected to begin validation of the Toxicological Screen based either on confirmed literature reports or experience in our laboratory such that the response of each compound in each biological endpoint of the screen was known and that overlap in toxicity occured among compounds 14,15,16,17,18,19. Concentrations for acrylamide and DES were selected following a dose range finding study performed in our laboratory. The dosages of other chemicals were based on either experience in our laboratory or on literature data from various oral short-term toxicity studies with the selected compounds. The highest dose was selected to produce frank toxicity while permitting sufficient survival for meaningful evaluation of the toxicity. The lower doses were than selected by inverse geometric progression.

ACRYLAMIDE

Acrylamide (Aldrich Chemical Co., Inc., Lot No. 14,866-0) was evaluated in the Tox Screen at exposure concentrations of 0, 3.75, 7.5, 15, 30 and 60 mg/kg rat. For SCE analysis, only concentrations of 15, 30 and 60 mg/kg were evaluated. As part of the neonatal survival experiment, dams were exposed to 15 mg/kg. Dosing solutions were prepared in water twice for both males and females during the 10-day treatment period and stored in the dark to insure the integrity of the samples. The concentration of each solution was confirmed by reverse phase HPLC.

CHLORDECONE

Chlordecone, obtained from the U.S. EPA, Pesticides and Industrial Chemicals Repository (Lot #E942), was dissolved in corn oil at concentrations suitable for exposure of 0, 0.625, 1.25, 2.5, 5.0 and 10.0 mg/kg body weight in rats. Concentrations of 0, 0.625, 2.5 and 10.0 mg/kg were utilized for SCE analysis. All males received 2 ml/kg of chlordecone. Dams were treated with 10.0 mg/kg chlordecone (~ 2.6 ml/kg). Due to the known stability of chlordecone, solutions were prepared only once for the males and once for the females. Compound identification was performed by gas chromatography. A Karl Fischer titration showed the water content to be approximately 11.54% H_2O.

CYCLOPHOSPHAMIDE

Cyclophosphamide (Sigma Chemical Co.) was evaluated in the Tox Screen at exposure concentrations of 0, 1.5, 3.0, 6.0, 12.0 and 24.0 mg/kg in rats. For SCE analysis only concentrations of 0, 1.5, 6.0 and 12.0 mg/kg were evaluated. As part of the neonatal survival experiment, dams were exposed to 12.0 mg/kg. In all three cases, 2 ml/kg was utilized as the exposure volume. Since cyclophosphamide is stable in solution only for 6 days solutions were prepared three different times during the 10-day treatment period for both the males and the dams[18]. All samples were prepared in water. The concentration of each solution was confirmed by reverse phase HPLC.

DIETHYLSTILBESTROL (DES)

DES, obtained from Aldrich Chem. Co, Inc. (Lot No. LK0718LK), was suspended in corn oil for dosing at 0, 31.25, 62.5, 125, 250 and 500 mg/kg in rats. Concentrations of 0, 31.25, 62.5 and 125 mg/kg were utilized for SCE analysis. Dams were treated with one half the lowest concentration used for

the males, 15 mg/kg. Solutions were prepared twice during the 10 day treatment period for both males and females and stored in the dark. Gentle heating of the solutions in a 40°C water bath was required immediately before dosing to increase the solubility of the compound. Confirmation of the concentrations of solutions were not performed due to the nature of the solvent.

SECTION 3

METHODS

The protocol for the Toxicological Screen can be divided into two sections. The first is the treatment protocol which defines the animal exposure regimen. The second is the biological screens which includes the toxicological studies performed with the whole animal or various body tissues and fluids (Figures 1 and 2). Only the logistics of performing the screen as a single protocol are described in this report.

ANIMAL HUSBANDRY

The subjects used in this study were male rats of the Fischer 344 strain (Charles River Breeding Laboratories) that were approximately 60 days of age on arrival. After a 7-9 day holding period, they were identified by ear tags, randomly assigned to treatment groups, and housed 2 per polycarbonate (Northeastern Products) cage with heat treated pine shavings as bedding. The animal housing room (class 100 clean room) was maintained at a desired 21° ± 0.6°C with the relative humidity at 50% ± 5%. A 12-hr light-dark cycle (light: 6 A.M. - 6 P.M.) was used. Food (Ralston Purina Lab Chow, #5001) and water were given ad lib.

THE TREATMENT PROTOCOL

Fischer 344 male rats were exposed by gavage to a pure chemical for 10 consecutive days. The 10 consecutive day exposure regime was selected based on literature data demonstrating the relationship between short- and long-term feeding studies[4,5]. This exposure regime was approved by the

peer review panel. Five concentrations of the test material and two negative control concentrations (cage and vehicle) were utilized. The maximum dosage delivered to the animal was based on literature reports for the chemical or dose range finding studies (see section III). The remaining 4 doses were reduced logarithimically, usually by factors of Log 2 or 3 (Figure 1). Exposure of the test material was adjusted according to the weight gain or loss of the animal. The animals were weighed on days 0, 3, 6 and 10 of the 10 day study. The dosing volume was calculated for each rat based on its latest weight.

Seven groups of animals were exposed to the wastes. The cage control and 2 of the 5 treatment groups each had 10 animals. The vehicle control group and 3 of the 5 treatment groups each had 14 animals/group, bringing the total number to 86 animals. The extra four animals/group in 4 of the groups were utilized for sister chromatid exchange analysis.

Toxicological Screen

Treatment Protocol

- One species: rats, Fischer 344
- One sex: males
- One age group: adults (70 days)
- One route of exposure: gavage
- Dose: 5 doses
- Length of treatment: 10 consecutive days

→ Teratology Protocol (based on Toxicological Screen results)

Biological Screens

General Toxicology	Mutagenesis/Carcinogenesis	Neurotoxicology	Immunotoxicology	Reproduction

• Death
• Weight change
• Organ/body weight ratios:
  ▪ Kidney
  ▪ Adrenals
  ▪ Liver
• Gross necropsy
• Clinical tests:
  ▪ Urinalysis
  ▪ Liver chemistry
  ▪ Serum chemistry
	• Bioassay of body fluids and tissues
	  ▪ Mutagens in urine
	  ▪ *In vivo* sister chromatid exchange in bone marrow
	• Direct bioassay of sample
	  ▪ Point mutation in bacteria
		• Motor activity: Figure 8 maze
		• Acoustic startle response
			• Hematology
			• Organ/body weight ratios:
			  ▪ Spleen
			  ▪ Thymus
			• Mitogen response
			• Natural killer cell activity
				• Testes weight
				• Sperm concentration
				• Sperm motility
				• Testosterone level
				• FSH levels
				• Testicular pathology

Figure 1

Teratology Protocol

- One species: rats, Fischer 344
- One sex: females
- One age group: adults
- One route of exposure: gavage
- Dose: 1 dose based on Toxicological Screen results
- Length of treatment: 10 consecutive days

Teratology Screen

- Neonatal survival
- SCE on maternal lymphocytes
- SCE on embryo livers
- Neurotoxicology screen on 3-week-old pups
- Immunotoxicology screen on 6-week-old rats

Teratology Portion of Toxicological Screen

Figure 2

BIOLOGICAL ENDPOINTS

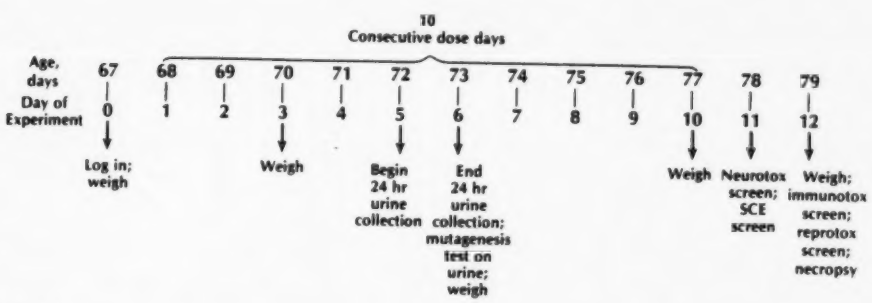
During or at the end of the 10 day treatment the whole animal and/or body tissues and fluids were evaluated in a series of toxicological assays. These assays are listed in Figures 1 and 2. A time-course description of the order in which these assays were performed is given below and in Figure 3.

Mutagens In The Urine

The mutagenic evaluation of urine from rats gavaged with the test chemicals was used to identify metabolic and physiological events which occur in the intact organism and which can not be duplicated *in vitro*. Body fluid analyses were performed in an attempt to identify the *in vivo* formation of mutagens[6]. A urine sample (or neat sample) is considered mutagenic if it produces a consistent dose-related increase over background values. Results from this study can play a critical role in determining the relative risk of genetic damage resulting from exposure to the test compounds.

Immediately following the administration of the 5th consecutive dose (day 5), twenty-four-hour urine samples were collected from one animal in each treatment group. The 7 animals were randomly selected and placed in 7 nalgene metabolism cages. Each cage was suspended over dry ice to ensure the integrity of the urine samples. Immediately following collection (day 6), the raw urine was evaluated for the presence of mutagens using the Ames Test. Each urine sample was evaluated using strains TA98 and TA100 at 3 to 5 concentrations (depending on sample size) in triplicate, both with and without a rat liver microsomal preparation.

Schematic Overview of the Toxicological Screen


10
Consecutive dose days
Age, days
Day of Experiment
67 68 69 70 71 72 73 74 75 76 77 78 79
0 1 2 3 4 5 6 7 8 9 10 11 12
Log in; weigh
Weigh
Begin 24 hr urine collection
End 24 hr urine collection; mutagenesis test on urine; weigh
Weigh
Neurotox screen; SCE screen
Weigh; immunotox screen; reprotox screen; necropsy

Teratology Screen

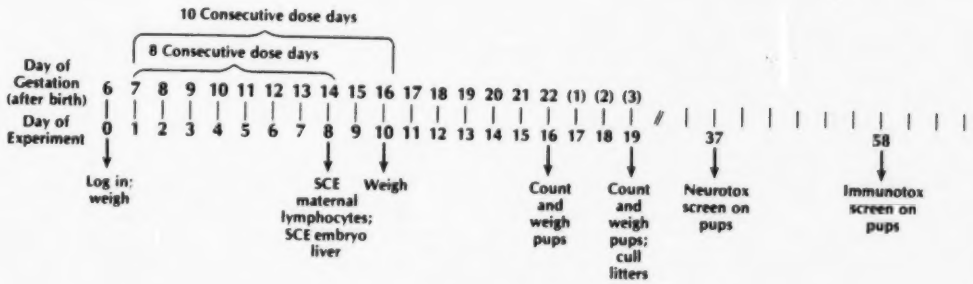

10 Consecutive dose days
8 Consecutive dose days
Day of Gestation (after birth)
Day of Experiment
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 (1) (2) (3)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 37 58
Log in; weigh
SCE maternal lymphocytes; SCE embryo liver
Weigh
Count and weigh pups
Count and weigh pups; cull litters
Neurotox screen on pups
Immunotox screen on pups

Figure 3

In Vivo Sister Chromatid Exchange (SCE)

Sister chromatid exchange analysis has become widely used as a sensitive measure of induced chromosomal alterations[7]. In the present study, *in vivo* methodological approaches were used to measure SCE frequencies in treated rat bone marrow cells.

On the morning following the 10th consecutive dose, four animals designated for SCE studies from three of the five treatment groups and the vehicle control group were pretreated with bromodeoxyuridine. These 16 animals were then given colchicine on day 11 followed by the harvesting of bone marrow cells for SCE analysis.

Neurotoxicology Screen

Because behavorial and neurological complaints are reported by people exposed to some industrial wastes, it is necessary to employ rapid neurotoxicological tests to provide an indication of the hazard posed by specific waste samples. The neurotoxic potential of the test compounds was assessed by routine short-term behavorial tests including motor activity and acoustic startle response[8,9]. These behavorial tests are among the most rapid and reliable tests of neurotoxicity.

Twenty-four hours after the 10th consecutive dose (day 11 of the study), 10 animals from each concentration group were evaluated in the neurotoxicological screen. This involved the assessment of motor activity using the figure 8 maze and response to auditory stimuli using the acoustic startle response test.

Remaining Screens

The following determinations were performed on each animal unless otherwise stated.

On day 12 the same 10 animals evaluated in the neurotoxicological screen were anesthetized, weighed, and exsanguinated through the abdominal aorta. A small portion of whole blood (0.5 ml) was placed in a test tube for leukocyte count determinations. A blood smear was also made for differential leukocyte counts. Serum was prepared from the remaining blood for liver function and other serum chemistry tests and for testosterone and follicle stimulating hormone (FSH) determinations. These parameters were used to assess specific and general somatic normality. In order to determine the immunotoxic potential of wastes, the thymus and spleen were removed aseptically and weighed. Spleen cells from each concentration group were utilized for mitogen response and natural killer (NK) cell assays. The mitogen assay is a good *in vitro* correlate of *in vivo* T-and B-lymphocyte function[10]. Natural killer cells are a nonspecific first line of defense against tumors, certain viruses, and certain infectious agents. The 4 hour ^{51}chromium release assay employed here for NK activity correlates very well with *in vivo* tumor model assays[11].

For an initial assessment of reproductive toxicity, the testes and epididymides were removed and weighed. The right testis from each animal was trimmed and placed in Bouin's fixative for pathological examination. The epididymal fluid from the left carda epididymis was utilized for studies of sperm concentration, motility and abnormal spermatoza. Sperm head counts were made with the left testis. These physiological measurements are common for assessment of risk in the male rat[12].

As part of the necropsy performed on day 12, urine was collected directly from the bladder with a sterile syringe and applied to an Ames' Bili-Labstix. Each animal was then examined for gross thoracic, abdominal, and external pathology including examination of the heart, lung, liver,

kidney, and adrenal. The liver, kidney, and adrenals were weighed and organ-to-body-weight ratios were calculated to assess specific organ toxicity and stress response. Except for the spleen, testes and epididymides, all animals and organs were discarded at the end of experimental day 12.

Teratology Screen (Neonatal Survival)

The neonatal survival screen, which was developed at HERL, is a simple, cost-effective test[13]. Its results are highly correlated with those obtained in the standard teratology protocol. It relies upon the well-known observation that rodent pups born with birth defects are generally cannibalized by the dam, and that those which do survive show significant growth deficits. These end points, death and weight loss, during the first 3 days postpartum are extremely easy to measure. This screen, therefore, provides a simple way to identify agents having the potential to adversely affect the embryo/fetus.

Forty-eight (24 treated; 24 controls) pregnant Fischer 344 female rats were exposed by gavage to one concentration of the compound beginning on day 7 of gestation and ending on day 16 (10 consecutive days). The selection of the single exposure concentration for neonatal survival was made near or at the maximum tolerable dose level observed for the males in the Tox Screen. The dosing volume was calculated based on the initial weight of the female on day 6 of gestation. This dosing volume was utilized for 10 consecutive days. The change in maternal weight during the treatment period was determined by weighing the dams after the 10th dose. The animals were allowed to give birth (day 22 of gestation) and the litters were counted and weighed at birth and at 3 days of age. The resulting litters were randomly reduced to one male and one female. At 3 weeks of age the pups were evaluated in the neurotox

screen; at 6 weeks the pups were killed for the immuno tox screen. This sequence is shown in Figures 2 and 3.

In Vivo Sister Chromatid Exchange

In order to determine if transplacental SCE induction occured with treatment of the dams with the test compounds, SCE frequencies in maternal lymphocytes and/or bone marrow cells as well as embryo liver cells were compared to control levels.

After the 8th consecutive dose, maternal blood was collected from the 8 animals designated for SCE analysis (4 treated, 4 control). The blood samples were cultured for lymphocyte SCE analysis. Immediately following blood removal, the dams were administered 5-bromodeoxyuridine. The following day the animals were killed and embryo livers were assessed for SCE frequencies. Alternatively, when maternal marrow cells were taken (instead of lymphocytes) for SCE analysis, the marrow was removed at the time of embryo removal, cultured for an additional 4-6 hours, and processed for SCE analysis.

SECTION 4

STATISTICAL METHODS USED IN EVAULATING TOX SCREEN RESULTS

In order to permit comparison of the results obtained with the 4 chemicals in the Tox Screen, a common general approach was taken for establishing the statistical significance of responses. This general approach was taken only when it was feasible and sensible with respect to the data and discipline. This approach involved the use of ANOVA followed by an appropriate T test using a p value of < 0.05.

Each researcher was allowed to select the method most appropriate for the analysis of his/her test results. However, advise concerning the use of methods for multiple comparisons, criteria for establishing p values, and references to cage or sham controls was provided by the project's statisticians. Where necessary the statisticians worked closely with the researchers to modify and correct the statistical procedures used. The following table (Table 1) lists the statistical methods used for each discipline of toxicology in the screen. The test methods listed provided the parameters for generating the calculated test results for all four compounds and can be referred to when examining the tables of results.

TABLE 1: STATISTICAL METHODS USED FOR THE TOXICOLOGICAL SCREEN

Criteria for Selection of Positive and Negative Responses.

Discipline of Toxicology	Statistical Method	Compared to Vehicle or Cage Control	p Value	Data Reported as
General Toxicology	ANOVA followed by Dunnett's t test	Vehicle	$p<.05$	Mean $\pm$ SE
Genetic Toxicology				
Bacterial mutagenesis	Non linear modeling of the Ames test	Vehicle	$p<.05$	Mean $\pm$ SE
SCE analysis	ANOVA followed by Dunnett's t test	Vehicle	$p<.05$	Mean $\pm$ SE
Neurotoxicology	ANOVA followed by Tukey's a test	Vehicle	$p<.05$	Mean $\pm$ SE
Immunotoxicology	ANOVA followed by Dunnett's t Test	Vehicle	$p<.05$	Mean $\pm$ SE
Reproductive Toxicology	ANOVA followed by Dunnett's t Test	Vehicle	$p<.05$	Mean $\pm$ SE
Teratology	ANOVA followed by Student's t test	Vehicle	$p<.05$	Mean $\pm$ SE

SECTION 5

RESULTS

The results of the Tox Screen with the 4 toxic chemicals are presented 1) by each discipline in narrative form and 2) as summary and data tables following each narrative. The summary tables list the results as positive (+) or negative (-) responses for each compound with each test at each dose level. The data tables list the data as a single number or as the mean with one standard error of the mean for each test at each dose level.

The following paragraphs give a narrative of the results for each compound in each discipline of toxicology in the Tox Screen.

A. GENERAL TOXICITY

The biological measures evaluated in general toxicology included body weight during and after dosing, lethality, weight of selected organs, organ/body weight ratios, urinalysis and serochemistry, a total of 39 variables. The measures were chosen on the basis of their frequent inclusion in screens for initial evaluation of toxic effects of chemicals. The summary of results and the data values for the general toxicity evaluations are given in Tables 2 to 6.

Acrylamide

Rats were dosed by gavage with aqueous acrylamide solutions at levels of 3.75-60 mg/kg, for 10 days The dosing routines were uneventful. Compared to shams (vehicle controls, 0 mg/kg), statistically different body weights were seen in the 60 mg/kg group as early as 3 days after dosing began. Acrylamide

at less than the highest dose did not appear to have any significant general effects on weights sacrifice or on serochemistries.

After the last dose, but before sacrifice, three rats in the high-dose group died. All of the survivors of that group had symptoms of inanition (no visceral body fat), which was consistent with the low body weight. These same rats had very distended bladders filled with urine. Two of these had a strong sweet odor to the carcass. The bladder distention is consistent with the literature and with the suspected neural paralytic toxicity of acrylamide.

Chlordecone

Administration of chlordecone resulted in no lethality at the highest dose (10 mg/kg), but most other variables were altered. Body weight was decreased 12% at the highest dose, and some of the other significant changes that were observed may have occurred because of this large body weight loss. Only the liver responded at the next lower dose (5 mg/kg), but its increased size was not confirmed by other measures. These results are consistent with the literature.

Cyclophosphamide

The results of the cyclophosphamide experiment revealed the lethal (70%) nature of the highest dose (24 mg/kg)(Table 2); survivors of this dose responded in all categories of test (Table 2), most likely because of the debilitating effect of this toxic compound. Body weight and serochemistries were altered at 12 mg/kg, and serochemistries, especially decreased alkaline phosphatase, at doses as low as 3 mg/kg. Decreased serum levels of this enzyme are not commonly observed in the rat. The other measured variables appeared to be consistent with data given in the literature.

Diethylstilbestrol

DES, even at the lowest dose used in this experiment (31.25 mg/kg), significantly decreased body and adrenal weights and cholesterol levels. The doses of DES used in this experiment may have been a hundred times higher than required to elicit changes.

The data in this study contain many inconsistencies when examined for dose-response relationships and when not corrected for inter-dependency of the measured variables; these inconsistencies probably reflect false negatives and false positives. When the variables are grouped in a way to accomodate for the interdependencies, many of these false negatives or positives may disappear. Also, correcting for these inter-dependecies may give us the ability to determine the toxicity of the chemical while reducing the number of measured variables.

Table 2: SUMMARY OF THE RESULTS OBTAINED IN GENERAL TOXICITY WITH THE TOXICOLOGICAL SCREEN

ACRYLAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	3.75 (10)	7.5 (10)	15.0 (10)	30.0 (10)	60 (10)
LETHALITY[a]	0	0	0	0	0	0	30%
BODY WEIGHT	-[b]	-	-	-	-	+	+
LIVER WEIGHT	-	-	-	-	-	-	NT[c]
LIVER/BODY	-	-	-	-	-	+	NT
KIDNEY WEIGHT	-	-	-	-	-	-	-
KIDNEY/BODY	-	-	-	-	-	-	+
ADRENAL WEIGHT	-	-	-	-	-	-	-
ADRENAL/BODY	-	-	-	-	-	-	+
URINALYSIS	-	-	-	-	-	-	NT
SERUM CHEMISTRY[d]	-	-	-	-	-	-	NT

CHLORDECONE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	0.625 (10)	1.25 (10)	2.5 (10)	5.0 (10)	10.0 (10)
LETHALITY	0	0	0	0	0	0	0%
BODY WEIGHT	-	-	-	-	-	-	+
LIVER WEIGHT	-	-	-	-	-	+	-
LIVER/BODY	-	-	-	-	-	+	+
KIDNEY WEIGHT	-	-	-	-	-	-	+
KIDNEY/BODY	-	-	-	-	-	-	-
ADRENAL WEIGHT	-	-	-	-	-	-	+
ADRENAL/BODY	-	-	-	-	-	-	+
URINALYSIS	-	-	-	-	-	-	-
SERUM CHEMISTRY	-	-	-	-	-	-	(6)

CYCLOPHOSPHAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	1.5 (10)	3.0 (10)	6.0 (10)	12.0 (10)	24.0 (10)
LETHALITY	0	0	0	0	0	0	70%
BODY WEIGHT	-	-	-	-	-	+	+
LIVER WEIGHT	-	-	-	-	-	-	+
LIVER/BODY	-	-	-	-	-	-	-
KIDNEY WEIGHT	-	-	-	-	-	-	-
KIDNEY/BODY	-	-	-	-	-	-	-
ADRENAL WEIGHT	-	-	-	-	-	-	+
ADRENAL/BODY	-	-	-	-	-	-	+
URINALYSIS	-	-	-	-	-	-	NT
SERUM CHEMISTRY	-	-	-	(1)	(1)	(5)	(8)

DES DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	31.25 (10)	62.50 (10)	125 (10)	250 (10)	500 (10)
LETHALITY	0	0	0	0	0	0	10%
BODY WEIGHT	-	-	+	+	+	+	+
LIVER WEIGHT	-	-	+	-	-	-	-
LIVER/BODY	-	-	+	+	+	+	+
KIDNEY WEIGHT	-	-	-	-	-	-	-
KIDNEY/BODY	-	-	+	+	+	+	+
ADRENAL WEIGHT	-	-	+	+	+	+	+
ADRENAL/BODY	-	-	+	+	+	+	+
URINALYSIS	-	-	NT	NT	-	-	-
SERUM CHEMISTRY	-	-	(3)	(5)	(5)	(7)	(6)

[a] %, LETHALITIES.
[b] -, NOT SIGNIFICANTLY DIFFERENT FROM CONTROLS; +, SIGNIFICANTLY DIFFERENT FROM CONTROLS.
[c] NT, NOT TESTED DUE TO LETHALITIES, INSUFFICIENT SAMPLE TISSUE, OR NOT SCHEDULED TO BE EVALUATED.
[d] TOTAL PARAMETERS EVALUATED = 21, ( ) INDICATES THE NUMBER OF PARAMETERS SIGNIFICANTLY DIFFERENT FROM CONTROLS.

Table 3: RESULTS OF ACYLAMIDE IN GENERAL TOXICITY WITH THE TOXICOLOGICAL SCREEN

GENERAL TOXICOLOGY		Units	DOSE: Mg/Kg 0	3.75	7.5	15.0	30.0	60.0
Body Weights	Day 0	grams	173	177	174	174	173	174
	3	grams	194	197	195	194	192	187
	6	grams	206	209	207	205	198[a]	177[a]
	10	grams	222	224	222	219	211[a]	155[a]
	12	grams	223	232	226	227	214	135[a]
Organ Weights	Adrenal	grams	0.0171	0.0162	0.0182	0.0179	0.0170	0.0197
	Adrenal/Body	%	0.0077	0.0070	0.0081	0.0079	0.0079	0.0146[a]
	Kidney	grams	0.840	0.879	0.869	0.880	0.835	0.625[a]
	Kidney/Body	%	0.377	0.379	0.384	0.387	0.389	0.463[a]
	Liver	grams	9.95	10.42	10.45	10.72	10.33	--
	Liver/Body	%	4.46	4.50	4.61	4.72	4.81	--
Urinalysis	pH		7.0	7.0	7.2	7.34	7.13	6.50[a]
Serum Chemistry	Glucose	mg/dl	155	160	157	163	157	NT[b]
	Sodium	mmole/l	131	134	132	131	135	NT
	Potassium	mmole/l	5.34	5.40	5.18	5.29	5.11	NT
	Chloride	mmole/l	94	95	92	95	95	NT
	BUN	mg/dl	20.1	20.7	20.4	20.8	20.9	NT
	Creatinine	mg/dl	-	-	-	-	-	NT
	BUN/Creatinine		41.8	45.8	46.1	48.7	50.5	NT
	Phosphate	mg/dl	7.5	7.7	7.7	7.3	7.6	NT
	Calcium	mg/dl	10.5	11.0	10.7	10.8	10.9	NT
	Cholesterol	mg/dl	-	-	-	-	-	NT
	Protein	gm/dl	5.8	6.0	5.9	6.0	5.9	NT
	Albumin	gm/dl	3.1	3.1	3.1	3.1	3.2	NT
	Globulin	gm/dl	2.8	2.9	2.8	2.9	2.8	NT
	A/G		1.1	1.1	1.1	1.1	1.0	NT
	TOT Bilirubin	mg/dl	0.28	0.26	0.25	0.26	0.25	NT
	DIR Bilirubin	mg/dl	0.05	0.05	0.04	0.04	0.05	NT
	ALK Phosp.	IU/l	129	128	135	128	128	NT
	GGTP	Units/l	8.2	7.7	7.9	7.3	8.2	NT
	SGPT	IU/l	52.8	54.5	50.4	57.2	63.0	NT
	SGOT	IU/l	82.9	84.0	82.6	81.1	78.9	NT
	LDH	IU/l	720	792	715	588	457	NT

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.

Table 4: RESULTS OF CHLORDECONE IN GENERAL TOXICITY WITH THE TOXICOLOGICAL SCREEN

GENERAL TOXICOLOGY		Units	DOSE: Mg/Kg 0	0.625	1.25	2.5	5.0	10.0
Body Weights	Day 0	grams	182	182	184	182	184	185
	3	grams	195	195	196	195	197	200[a]
	6	grams	206	206	207	206	207	209
	10	grams	221	222	218	220	213	203[a]
	12	grams	222	228	229	230	227	199[a]
Organ Weights	Adrenal	grams	0.0203	0.0194	0.0207	0.0206	0.0207	0.0232[a]
	Adrenal/Body	%	0.0090	0.0054	0.0091	0.0089	0.0096	0.0124[a]
	Kidney	grams	0.841	0.827	0.855	0.849	0874	0.71[a]
	Kidney/Body	%	0.372	0.363	0.373	0.368	0.385	0.379
	Liver	grams	9.82	9.83	10.24	10.51	11.36[a]	9.49
	Liver/Body	%	4.34	4.32	4.55	4.56	5.01[a]	5.03[a]
Urinalysis	pH		7.38	7.43	7.30	7.31	7.50	7.40
Serum Chemistry	Glucose	mg/dl	169	175	167	173	156	133[a]
	Sodium	mmole/l	137	135	135	136	135	136
	Potassium	mmole/l	5.22	5.14	5.28	5.06	5.13	4.84
	Chloride	mmole/l	99	98	100	99	95	100
	BUN	mg/dl	20.8	21.2	20.3	21.6	20.3	26.1[a]
	Creatinine	mg/dl	.43	.41	.41	.41	.51	.51
	BUN/Creatinine		52.5	56.6	54.4	55.9	43.0	53.7
	Phosphate	mg/dl	8.1	7.7	7.8	7.8	7.9	7.1
	Calcium	mg/dl	10.8	10.8	10.8	10.8	10.9	10.1
	Cholesterol	mg/dl	32.5	32.2	33.2	32.3	29.0	10.4[a]
	Protein	gm/dl	6.0	6.0	6.0	6.1	6.0	5.9
	Albumin	gm/dl	3.2	3.2	3.2	3.2	3.2	3.2
	Globulin	gm/dl	2.9	2.8	2.8	2.9	2.8	2.6
	A/G		1.1	1.1	1.2	1.1	1.2	1.2
	TOT Bilirubin	mg/dl	0.30	0.29	0.31	0.28	0.28	0.19
	DIR Bilirubin	mg/dl	0.18	0.17	0.17	0.15	0.15	0.14
	ALK Phosp.	IU/l	110	108	110	122	119	130[a]
	GGTP	Units/l	4.4	3.2	3.8	3.6	4.2	7.6[a]
	SGPT	IU/l	44.4	43.9	44.4	47.7	47.9	89.3[a]
	SGOT	IU/l	74.5	72.0	65.5	65.0	62.3	83.5
	LDH	IU/l	358	262	216	152	236	136

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.

Table 5: RESULTS OF CYCLOPHOSPHAMIDE IN GENERAL TOXICITY WITH THE TOXICOLOGICAL SCREEN

GENERAL TOXICOLOGY		Units	DOSE: Mg/Kg 0	1.5	3.0	6.0	12.0	24.0
Body Weights	Day 0	grams	195	198	199	198	194	196
	3	grams	209	210	208	209	201[a]	201[a]
	6	grams	215	215	215	212	204[a]	202[a]
	10	grams	228	227	224	224	210[a]	173[a]
	12	grams	232	231	229	229	213[a]	169[a]
Organ Weights	Adrenal	grams	0.0193	0.0195	0.0194	0.0199	0.0204	0.0250[a]
	Adrenal/Body	%	0.008	0.0084	0.0085	0.0087	0.0096[a]	0.0149[a]
	Kidney	grams	0.866	0.867	0.869	0.858	0.817	0.788
	Kidney/Body	%	0.373	0.375	0.379	0.374	0.383	0.466
	Liver	grams	9.79	9.85	9.84	9.99	9.53	7.41[a]
	Liver/Body	%	4.22	4.25	4.29	4.36	4.472	4.37
Urinalysis	pH		7.10	7.38	7.25	7.36	7.70	NT[b]
Serum Chemistry	Glucose	mg/dl	168	163	167	163	161	157
	Sodium	mmole/l	135	135	135	134	130	134
	Potassium	mmole/l	4.79	5.05	5.05	4.96	4.37	4.57
	Chloride	mmole/l	85	95	94	96	93	97
	BUN	mg/dl	23.7	22.8	23.7	22.6	22.3	18.3[a]
	Creatinine	mg/dl	.47	.47	.49	.50	.48	.47
	BUN/Creatinine		50.1	49.2	48.6	45.3	47.3	40.0
	Phosphate	mg/dl	6.9	7.8	7.6	7.3	6.4	5.4
	Calcium	mg/dl	10.3	10.3	10.3	10.2	9.9	9.2[a]
	Cholesterol	mg/dl	41.7	34.6	31.6	39.2	33.9	22.0
	Protein	gm/dl	6.1	6.2	6.2	5.9	5.5[a]	4.8[a]
	Albumin	gm/dl	3.3	3.2	3.3	3.2	3.0[a]	2.1[a]
	Globulin	gm/dl	2.9	2.99	2.9	2.8	2.5[a]	2.7
	A/G		1.1	1.1	1.2	1.5	1.2	0.8[a]
	TOT Bilirubin	mg/dl	0.30	0.28	0.33	0.32	0.26	0.13
	DIR Bilirubin	mg/dl	.05	.05	.05	.05	.07	.05
	ALK Phosp.	IU/l	100	95	76.3[a]	66.4[a]	56.1[a]	20.0[a]
	GGTP	Units/l	48	4.7	4.2	4.6	7.8	3.0
	SGPT	IU/l	41.5	39.5	36.8	34.7	34.0	20.7[a]
	SGOT	IU/l	134	118	81	97	131	24
	LDH	IU/l	721	742	690	442	136[a]	46[a]

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.

Table 6: RESULTS OF DES IN GENERAL TOXICITY WITH THE TOXICOLOGICAL SCREEN

GENERAL TOXICOLOGY		Units	DOSE: Mg/Kg					
			0	31.25	62.5	125	250	500
Body Weights	Day 0	grams	180	173	168[a]	169[a]	172[a]	172[a]
	3	grams	192	190	183[a]	186[a]	183[a]	176[a]
	6	grams	194	176[a]	173[a]	173[a]	172[a]	153[a]
	10	grams	209	162[a]	165[a]	165[a]	169[a]	140[a]
	12	grams	212	145[a]	157[a]	164[a]	171[a]	144[a]
Organ Weights	Adrenal	grams	0.0206	0.344[a]	0.285[a]	0.295[a]	0.277[a]	0.292[a]
	Adrenal/Body	%	0.0097	0.0239[a]	0.0184[a]	0.0181[a]	0.0162[a]	0.0208[a]
	Kidney	grams	0.807	0.724	0.743	0.742	0.777	0.866
	Kidney/Body	%	0.379	0.49[a]	0.475[a]	0.454[a]	0.453[a]	0.603[a]
	Liver	grams	9.95	7.41[a]	9.58	10.52	11.34	10.26
	Liver/Body	%	4.68	5.09[a]	6.10[a]	6.43[a]	6.49[a]	7.06[a]
Urinalysis	pH		7.25	7.00	6.50	7.00	7.33	7.13
Serum Chemistry	Glucose	mg/dl	151	120[a]	128[a]	126[a]	125[a]	133
	Sodium	mmole/l	135	136	135	136	136	136
	Potassium	mmole/l	4.88	4.71	4.91	4.62	4.78	4.81
	Chloride	mmole/l	96	97	95	96	96	95
	BUN	mg/dl	20.1	17.0	17.6	18.5	17.5	29.3[a]
	Creatinine	mg/dl	.41	.37	.46	.44	.46	.60[a]
	BUN/Creatinine		49.7	46.3	39.2	42.2	38.6	52.3
	Phosphate	mg/dl	7.8	6.1[a]	6.9	6.7	6.5[a]	6.7[a]
	Calcium	mg/dl	10.6	9.9	10.4	10.7	10.7	10.4
	Cholesterol	mg/dl	44.9	8.2[a]	28.0[a]	23.1	23.1[a]	41.0
	Protein	gm/dl	6.4	6.5	6.9[a]	7.1[a]	7.0[a]	6.7
	Albumin	gm/dl	3.1	3.0	3.1	3.2	3.3	3.2
	Globulin	gm/dl	3.2	3.5	3.8[a]	3.8[a]	3.7[a]	3.6
	A/G		0.99	0.87	0.81[a]	0.84[a]	0.87	0.90
	TOT Bilirubin	mg/dl	0.30	0.13	0.22	0.27	0.29	0.53[a]
	DIR Bilirubin	mg/dl	0.13	0.07	0.10	0.11	0.12	0.20
	ALK Phosp.	IU/l	99	109	107	99	100	67
	GGTP	Units/l	4.5	5.1	8.1	10.1	14.4[a]	24.9[a]
	SGPT	IU/l	37.8	109	180	186[a]	168[a]	141[a]
	SGOT	IU/l	65.3	79.6	94.8	102	91.7	86.7
	LDH	IU/l	572	330	326	316	304	249

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.

B. GENETIC TOXICOLOGY

The genetic toxicology tests used for the validation study of the Tox Screen included several genetic test systems and several genetic endpoints. The tests included measurement of gene mutations in the bacterium Salmonella typhimurium and the measurement of sister chromatid exchanges (SCE) in male rat bone marrow and/or maternal and fetal liver[20]. The four compounds used for the validation study were tested in the Salmonella assay in the presence or absence of metabolic activation (rat liver S9). In addition, the urine from rats treated with the compounds was also tested for the presence of mutagenic metabolites in the Salmonella assay. SCEs were measured in the bone marrow cells of rats and also in the liver cells of pregnant female rats and their fetuses. The results are described below and listed in Tables 7 to 9; (SCE results in dams and embryo cells are listed in Tables 16 to 18 for each of the four compounds). The data suggest that the procedures used to detect genetic toxicity of the four compounds are useful and effective.

Acrylamide

Acrylamide was not mutagenic in Salmonella strains TA98 and TA100 either with and without metabolic activation. The urine of acrylamide-treated rodents was not mutagenic. Acrylamide did, however, induce SCEs in the bone marrow of male rats at all three concentrations tested (15, 30, and 60 mg/kg/day). Cell replication kinetics data did not reveal significant cytotoxic effects. SCEs were not induced by acrylamide in maternal lymphocytes or in fetal liver of preganant rats. These results suggest that acrylamide may not cause gene mutations but may cause chromosomal effects. No information was available in the literature regarding the genetic toxicity of acrylamide

prior to this study, and these data indicate that acrylamide may possess some genetic toxicity.

Chlordecone

Both chlordecone and the urine of chlordecone-treated rats were not mutagenic in Salmonella strains TA98, TA100, or TA102 with and without Aroclor-induced rat liver homogenate[21]. In addition, chlordecone did not induce SCEs in either male bone marrow cells or in liver cells of pregnant female rats or their fetuses at any concentration tested (0.625, 2.5, and 10 mg/kg/day). There is no information in the literature regarding the mutagenicity of chlordecone. Because chlordecone is a chlorinated hydrocarbon pesticide, it would not be expected to be mutagenic in the Salmonella assay or induce SCEs[22,23]. Our results are consistent with this expectation.

Cyclophosphamide

Cyclophosphamide was clearly mutagenic in Salmonella. The urine of cyclophosphamide-treated rodents was also mutagenic when evaluated in strains TA1535 and TA100. The addition of exogenous metabolic activation had very little effect on mutagenicity; this fact implies that cyclophosphamide is metabolized by the host tissue and that the ultimate mutagenic forms are excreted through the urine. In addition, for the 3 concentrations tested (1.5, 6.0, and 12 mg/kg/day) cyclophosphamide induced up to a 15-fold increase in SCEs over background values in bone marrow cells [24,25]. It was not tested for its ability to induce SCEs in maternal lymphocytes or fetal liver cells due to the total resorbtion of the pups at the concentration evaluated (12 mg/kg/day). Cyclophosphamide is a well-known alkylating agent and antitumor compound. These results are consistent with the literature and indicate that the tests are sensitive to alkylating agents, which would be expected to be genotoxic.

Diethylstilbestrol

Diethylstilbestrol (DES) was not mutagenic in Salmonella nor was the urine of DES-treated rodents mutagenic when evaluated in strains TA100 and TA98 in the presence and absence of a metabolizing system. DES did not induce SCEs in male bone marrow at any of the doses tested (31.25, 62.5, and 125 mg/kg/day). Similarly, there was no effect on the cell replication kinetics in the various treatment groups. It was not tested for its ability to induce SCEs in maternal and fetal liver cells due to the lack of surviving pups at the concentration tested for neonatal survival (31.25 mg/kg/day). Based on studies in the literature, DES was not expected to be mutagenic in Salmonella. However, some studies have reported that DES causes SCEs in bone marrow.

TABLE 7: SUMMARY OF RESULTS OBTAINED IN MUTAGENESIS WITH THE TOXICOLOGICAL SCREEN

ACRYLAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control	Vehicle Control	3.75 (10)	7.5 (10)	15.0 (14)	30.0 (14)	60 (14)
MUTAGENS IN URINE	-[a]	-	-	-	-	-	-
SCE BONE MARROW	NT[b]	-	NT	NT	+	+	+
IN-VITRO AMES TEST	NEGATIVE						

CHLORDECONE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control	Vehicle Control	0.625 (14)	1.25 (10)	2.5 (14)	5.0 (10)	10.0 (14)
MUTAGENS IN URINE	-	-	-	-	-	-	-
SCE BONE MARROW	NT	-	-	NT	-	NT	-
IN-VITRO AMES TEST	NEGATIVE						

CYCLOPHOSPHAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control	Vehicle Control	1.5 (14)	3.0 (10)	6.0 (14)	12.0 (14)	24.0 (14)
MUTAGENS IN URINE	-	-	+	+	+	+	+
SCE BONE MARROW	NT	-	+	NT	+	+	NT
IN-VITRO AMES TEST	POSITIVE						

DES DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control	Vehicle Control	31.25 (14)	62.50 (14)	125 (14)	250 (10)	500 (10)
MUTAGENS IN URINE	-	-	-	NT	-	-	-
SCE BONE MARROW	NT	-	-	-	-	NT	NT
IN-VITRO AMES TEST	NEGATIVE						

[a]-, NOT SIGNIFICANTLY DIFFERENT FROM CONTROLS; +, SIGNIFICANTLY DIFFFERENT FROM CONTROLS.
[b]NT, NOT TESTED DUE TO LETHALITES, INSUFFICIENT SAMPLE TISSUE, OR NOT SCHEDULED TO BE EVALUATED.

Table 8: RESULTS OBTAINED WITH ACRYLAMIDE AND CHLORDECONE IN MUTAGENESIS WITH THE TOXICOLOGICAL SCREEN

ACRYLAMIDE	Salmonella Strains[c]	Units	DOSE: Mg/Kg 0	3.75	7.5	15.0	30.0	60.0
Mutagens in Urine	TA1535 - S9	Rev/100ul	NT[b]	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	TA98 - S9	Rev/100ul	7.1	3.6	5.2	7.8	8.6	5.2
	+ S9	Rev/100ul	10.2	5.9	7.5	9.6	11.5	7.7
	TA100 - S9	Rev/100ul	23.3	15.2	20.9	26.7	21.6	18.5
	+ S9	Rev/100ul	21.6	18.8	18.8	25.5	22.2	19.4
	TA102 - S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
SCE Analysis	Bone Marrow	SCE/cell (X ± SE)	3.28±0.49	NT	NT	4.7±0.29[a]	6.0±0.94[a]	14.1±0.99[a]

CHLORDECONE	Salmonella Strains[c]	Units	DOSE: Mg/kg 0	0.625	1.25	2.5	5.0	10.0
Mutagens in Urine	TA1535 - S9	Rev/100ul	NT[b]	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	TA98 - S9	Rev/100ul	5.6	5.0	4.5	4.7	4.3	3.9
	+ S9	Rev/100ul	5.1	4.9	4.7	5.0	6.3	2.8
	TA100 - S9	Rev/100ul	23.2	22.4	22.1	22.2	21.7	18.3
	+ S9	Rev/100ul	21.2	21.9	17.5	23.1	18.6	16.6
	TA102 - S9	Rev/100ul	47.8	50.1	55.9	57.2	57.1	46.0
	+ S9	Rev/100ul	61.5	58.8	63.4	59.7	61.8	53.8
SCE Analysis	Bone Marrow	SCE/cell (X ± SE)	3.0±0.37	3.5±0.2	NT	3.1±0.2	NT	3.4±0.63

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.
[c]SALMONELLA STRAINS WITH (+S9) AND WITHOUT (-S9) RAT LIVER HOMOGENATE.

Table 9: RESULTS OBTAINED WITH CYCLOPHOSPHAMIDE AND DIETHYLSTILBESTROL IN MUTAGENESIS WITH THE TOX SCREEN

CYCLOPHOSPHAMIDE	Salmonella Strain[c]	Units	DOSE: Mg/Kg 0	1.5	3.0	6.0	12.0	24.0
Mutagens in Urine	TA1535 - S9	Rev/100ul	8.1	17.7	26.3	30.4	64.5	74.2
	+ S9	Rev/100ul	3.2	15.3	25.5	34.5	75.5	85.2
	TA98 - S9	Rev/100ul	NT[b]	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	TA100 - S9	Rev/100ul	14.4	19.1	20.1	26.4	52.6	57.6
	+ S9	Rev/100ul	15.4	27.1	27.8	31.6	61.6	70.7
	TA102 - S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
SCE Analysis	Bone Marrow	SCE/cell (X ± SE)	3.0±0.65	8.4±1.2[a]	NT	20.6±3.4[a]	45.3±2.5[a]	NT

DIETHYLSTILBESTROL	Salmonella Strains[c]	Units	DOSE: Mg/Kg 0	31.25	62.5	125	250	500
Mutagens in Urine	TA1535 - S9	Rev/100ul	NT[b]	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	TA98 - S9	Rev/100ul	9.9	2.1	NT	47.5	2.3	3.2
	+ S9	Rev/100ul	12.3	3.3	NT	2.6	4.5	3.3
	TA100 - S9	Rev/100ul	16.6	22.5	31.1	14.6	16.6	18.1
	+ S9	Rev/100ul	23	22.8	9.1	14.3	13.8	22.8
	TA102 - S9	Rev/100ul	NT	NT	NT	NT	NT	NT
	+ S9	Rev/100ul	NT	NT	NT	NT	NT	NT
SCE Analysis	Bone Marrow	SCE/cell (X ± SE)	2.8±0.83	3.1±0.21	3.3±0.81	3.5±0.72	NT	NT

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.
[c]SALMONELLA STRAINS WITH (+S9) AND WITHOUT (-S9) RAT LIVER HOMOGENATE.

C. NEUROTOXICOLOGY

Evaluation of neurotoxicity was accomplished using motor activity and the acoustic startle response. Motor activity was assessed in figure eight mazes. The acoustic startle response was assessed by presenting a loud (120 dB, 13kHz) tone at each of three background noise intensities. Tests of motor activity and the acoustic startle response are among the most rapid and reliable tests of neurotoxicity, and have been used to assess the effects of a broad range of toxicants including pesticides, solvents, drugs and heavy metals. Behavioral determinations were made 24 hours after the tenth consecutive daily treatment with a toxicant, during one-hour (figure-eight mazes) or twenty-minute (acoustic startle response) sessions. The results of these experiments are described below and given in Tables 10 to 12 for each of the toxicants. Collectively, the results indicate that the tests are sensitive, specific and predictive of the neurotoxic potential of the four chemicals studied under the present conditions of exposure.

Acrylamide

Acrylamide produced dosage-related decreases in motor activity. Decreases in 1-hr motor activity of 12, 28, or 53%, measured in the figure-eight maze, were observed following ten daily dosages of 15, 30, or 60 mg/kg respectively. In addition, amplitude of the acoustic startle response was decreased following dosages of 30 or 60 mg/kg/day. An increased latency response was seen only at the largest dosage. Acrylamide is a widely recognized neurotoxicant that produces motor and sensory impairments. The results obtained in this experiment are therefore consistent with expectations, and indicate that these behavioral measures can be used to evaluate acrylamide neurotoxicity.

Chlordecone

Exposure of adult rats to chlordecone produced changes in both motor activity and sensorimotor reactivity. Motor activity in the figure-eight maze was decreased at the highest dosage of chlordecone, 10 mg/kg/day. This effect was present at 5 minutes of testing and persisted throughout the one-hour test session. In contrast to this hypoactivity, the amplitude of the acoustic startle response was increased by the two largest dosages, while latency of the response was unaffected by chlordecone. Chlordecone's effects were obtained in the absence of mortality. These results suggest that the behavioral effects of chlordecone represent neurotoxic actions of the compound. Chlordecone is a chlorinated hydrocarbon pesticide whose primary target is the nervous system. Chlordecone intoxication is characterized by tremors and hyperexcitability. An exaggerated startle response has been previously reported and is consistent with the startle data reported here[26,27,28].

Cyclophosphamide

Cyclophosphamide produced decreases in motor activity and sensorimotor reactivity at the highest dosage only, 24 mg/kg/day. Motor activity in the figure-eight maze was decreased by 65%; this effect was present at 5 minutes and persisted throughout the 1-hour test session. In addition, cyclophosphamide decreased amplitude and increased latency of the acoustic startle response, but only at the highest dosage. Since this dosage produced 50% mortality, it is likely that these behavioral changes reflect a general debilitation of the animals rather than specific neurotoxicity. Cyclophosphamide is an alkylating agent which would not be expected to adversely affect the adult nervous system[29]. The results of this study are therefore consistent with the general lack of neurotoxic potential of cyclophosphamide.

Diethylstilbestrol. Diethylstilbestrol produced uniform decreases in motor activity after 10 daily doses of 62.5 mg/kg and above. In addition, amplitude of the acoustic startle response was uniformly decreased and latency was uniformly increased at all dosages. The lack of dosage-related effects, together with the results of the general toxicity evaluations, suggest that debilitation of the animals rather than specific neurotoxicity was responsible for these behavioral effects. Diethylstilbestrol is a nonsteroidal synthetic estrogen that, while producing permanent changes in brain development, has very few effects on the mature nervous system[30]. The results of this experiment are therefore consistent with expectations regarding the general lack of neurotoxic potential of diethylstilbestrol.

TABLE 10: SUMMARY OF RESULTS IN NEUROTOXICOLOGY WITH THE TOXICOLOGIAL SCREEN

ACRYLAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	3.75 (10)	7.5 (10)	15.0 (10)	30.0 (10)	60 (10)
FIGURE-EIGHT MAZE	-[a]	-	-	-	+	+	+
ACOUSTIC STARTLE	-	-	+	-	+	+	+

CHLORDECONE DOSAGE: MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	0.625 (10)	1.25 (10)	2.5 (10)	5.0 (10)	10.0 (10)
FIGURE-EIGHT MAZE	-	-	-	-	-	-	+
ACOUSTIC STARTLE	-	-	-	-	+	+	+

CYCLOPHOS-PHAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	1.5 (10)	3.0 (10)	6.0 (10)	12.0 (10)	24.0 (10)
FIGURE-EIGHT MAZE	-	-	-	-	-	-	+
ACOUSTIC STARTLE	-	-	-	-	-	-	+

DES DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	31.25 (10)	62.50 (10)	125 (10)	250 (10)	500 (10)
FIGURE-EIGHT MAZE	-	-	-	+	+	+	+
ACOUSTIC STARTLE	-	-	+	+	+	+	+

[a]-, NOT SIGNIFICANTLY DIFFERENT FROM CONTROLS; +, SIGNIFICANTLY DIFFFERENT FROM CONTROLS.

Table 11: RESULTS OBTAINED WITH ACRYLAMIDE AND CHLORDECONE IN NEUROTOXICOLOGY WITH THE TOXICOLOGICAL SCREEN

ACRYLAMIDE		Units	Mg/Kg 0	3.75	7.5	15.0	30.0	60.0
Figure-8-Maze Activity		Counts/Hour (X ± SE)	253± 7	261±14	245±9	221±9[a]	181±9[a]	118±7[a]
Acoustic Startle Amplitude/ Bkg. Noise Condition	45 dB(A)	Grams of Force (X ± SE)	110±10	69±10[a]	111±12	80±11[a]	40±3[a]	21±8[a]
	60 dB(A)		145±9	87±9[a]	138±14	111±12[a]	62±5[a]	17±2[a]
	75 dB(A)		156±9	119±14[a]	148±15	123±17[a]	63±3[a]	25±4[a]
	Combined dB(A)		-	-	-	-	-	-

CHLORDECONE		Units	Mg/Kg 0	0.625	1.25	2.5	5.0	10.0
Figure-8-Maze Activity		Counts/Hour (X ± SE)	262±10	264±10	245±13	242±14	268±18	194±14[a]
Acoustic Startle Amplitude/ Bkg. Noise condition	50 dB(A)	Grams of Force (X ± SE)	122±13	140±15	114±14	143±14	199±15[a]	217±20[a]
	65 dB(A)		139±13	172±19	150±17	167±15	241±18[a]	250±21[a]
	80 dB(A)		152±14	164±21	150±12	204±16[a]	220±20[a]	212±25[a]
	Combined dB(A)		-	-	-	-	-	-

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.

Table 12: RESULTS OF CYCLOPHOSPHAMIDE AND DES IN NEUROTOXICOLOGY WITH THE TOXICOLOGICAL SCREEN

CYCLOPHOSPHAMIDE		Units	Mg/Kg 0	1.5	3.0	6.0	12.0	24.0
Figure-8-Maze Activity		Counts/Hour (X ± SE)	263±10	266±12	280±14	248±14	247±11	72±15[a]
Acoustic Startle Amplitude/ Bkg. Noise Condition	50 dB(A)	Grams of Force (X ± SE)	NT[b]	NT	NT	NT	NT	NT
	65 dB(A)		NT	NT	NT	NT	NT	NT
	80 dB(A)		NT	NT	NT	NT	NT	NT
	Combined dB(A)		127±7	151±9	123±9	115±8	119±9	75±7[a]

DIETHYLSTILBESTROL		Units	Mg/Kg 0	31.25	62.5	125	250	500
Figure-8-Maze Activity		Counts/Hour (X ± SE)	192±8	160±13	150±9[a]	148±5[a]	147±14[a]	134±16[a]
Acoustic Startle Amplitude/ Bkg. Noise Condition	45 dB(A)	Grams of Force (X ± SE)	NT	NT	NT	NT	NT	NT
	60 dB(A)		NT	NT	NT	NT	NT	NT
	75 dB(A)		NT	NT	NT	NT	NT	NT
	Combined dB(A)		100±6	44±4	49±4	51±4	43±4[a]	35±4[a]

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.

D. IMMUNOTOXICOLOGY

Several selected immune functional parameters were evaluated in Fischer 344 male rats following ten days of oral dosing with acrylamide, chlordecone, cyclophosphamide (CY) or diethylstilbestrol (DES). Two days after the tenth and final dosing, rats were evaluated for the following: 1) body and lymphoid (spleen and thymus) organ weights; 2) peripheral blood leukocyte counts; 3) *in vitro* mitogen response of splenocytes to phytohemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), which are T-cell mitogens; and *S. typhimurium* mitogen (STM), which is a B-cell mitogen; and 4) natural killer (NK) cell activity against allogeneic W/FU-G1 rat lymphoma target cells and xenogeneic YAC-1 mouse lymphoma target cells. Results are given in Tables 13 to 15.

Acrylamide

Exposure of rats to acrylamide did not produce immunotoxic effects in the absence of overt general toxicity. Only seven of ten rats given the highest dosage (60 mg/kg/day) of acrylamide survived. These rats had significantly decreased body, spleen and thymus weights and decreased peripheral blood leukocyte and lymphocyte counts. While mitogen responses and NK activity were depressed (60 mg/kg/day dosage) they were not found to be statistically significant compared with vehicle controls. These results suggest that acrylamide is not a direct immunotoxicant under the conditions of exposure in this study.

Chlordecone and Cyclophosphamide

All of the parameters examined were significantly affected in rats dosed with the known immunosuppressive drug cyclophosphamide[31,32]. Spleen and thymus weights were significantly ($p<0.05$) reduced at all dosages of

cyclophosphamide, while body weight loss was observed only at the two highest dosages (12 and 24 mg/kg/day). Total leukocyte counts and absolute lymphocyte counts were significantly reduced at all cyclophosphamide dosages. Significantly ($P<0.01$) reduced mitogen responses to PHA, Con A, PWM, and STM were observed at all cyclophosphamide dosages[31,33,34,35]. NK cell activity was significantly suppressed at 6 and 12 mg/kg/day[36,37]. Rats dosed with chlordecone displayed decreased lymphoid organ and body weights, leukocyte counts, NK activity and Con A responsiveness only at the highest dosage (10 mg/kg/day). It was concluded that these results represent an overt toxic response to chlordecone at this dosage, as reported in the literature.[38].

Diethylstilbestrol

Rats exposed to the synthetic estrogen, DES, at dosages of greater than 31.25 mg/kg/day had little effect on the immune parameters investigated except for significant losses in body and lymphoid organ weights. Spleen, thymus and body weights were significantly reduced at all dosages employed. Thymus weight to body weight ratios were significantly reduced at all dosages; however, spleen to body weight ratios, while reduced at all DES dosages, were not significantly different. This suggests that the decrease in body and spleen weight, but not thymus weight, may have resulted from water loss. These decreases, however, were not reflected in consistent significant changes in leukocyte counts, mitogen responsiveness or NK activity. The failure to observe alterations in the mitogen response and NK cell activity of DES treated male rats may be due to a sex and/or species effect, since it has been reported that female mice dosed with DES displayed reduced mitogen responses and NK activity[39,40].

TABLE 13: SUMMARY OF RESULTS OBTAINED IN IMMUNOLOGY WITH THE TOXICOLOGICAL SCREEN

ACYRLAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	3.75 (10)	7.5 (10)	15.0 (10)	30.0 (10)	60 (10)
LEUKOCYTE COUNT	-[a]	-	-	-	-	-	+
SPLEEN WEIGHT	-	-	-	-	+	+	+
SPLEEN/BODY	-	-	-	-	+	+	+
THYMUS WEIGHT	-	-	-	-	-	+	+
THYMUS/BODY	-	-	-	-	-	-	+
MITOGEN RESPONSE	-	-	-	-	-	-	+
NATURAL KILLER CELL ACTIVITY	-	-	-	-	-	-	+

CHLORDECONE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	0.625 (10)	1.25 (10)	2.5 (10)	5.0 (10)	10.0 (10)
LEUKOCYTE COUNT	-	-	-	-	-	-	+
SPLEEN WEIGHT	-	-	-	-	-	-	+
SPLEEN/BODY	-	-	-	-	-	-	+
THYMUS WEIGHT	-	-	-	-	-	-	+
THYMUS/BODY	-	-	-	-	-	-	+
MITOGEN RESPONSE	-	-	-	-	+	+	+
NATURAL KILLER CELL ACTIVITY	-	-	-	-	-	-	+

CYCLOPHOSPHAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	1.5 (10)	3.0 (10)	6.0 (10)	12.0 (10)	24.0 (10)
LEUKOCYTE COUNT	-	-	+	+	+	+	+
SPLEEN WEIGHT	-	-	+	+	+	+	+
SPLEEN/BODY	-	-	+	+	+	+	+
THYMUS WEIGHT	-	-	+	+	+	+	+
THYMUS/BODY	-	-	+	+	+	+	+
MITOGEN RESPONSE	-	-	+	+	+	NT[b]	NT
NATURAL KILLER CELL ACTIVITY	-	-	-	-	+	+	NT

DES DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (10)	Vehicle Control (10)	31.25 (10)	62.50 (10)	125 (10)	250 (10)	500 (10)
LEUKOCYTE COUNT	-	-	-	-	-	-	+
SPLEEN WEIGHT	-	-	+	+	+	+	+
SPLEEN/BODY	-	-	-	-	-	-	-
THYMUS WEIGHT	-	-	+	+	+	+	+
THYMUS/BODY	-	-	+	+	+	+	+
MITOGEN RESPONSE	-	-	+	+	-	-	+
NATURAL KILLER CELL ACTIVITY	-	-	-	-	-	-	+

[a]-, NOT SIGNIFICANTLY DIFFERENT FROM CONTROLS; +, SIGNIFICANTLY DIFFFERENT FROM CONTROLS.
[b]NT, NOT TESTED DUE TO LETHALITES, INSUFFICIENT SAMPLE TISSUE, OR NOT SCHEDULED TO BE EVALUATED.

Table 14: RESULTS OBTAINED WITH ACRYLAMIDE AND CHLORDECONE IN IMMUNOLOGY WITH THE TOXICOLOGICAL SCREEN

ACRYLAMIDE		Units	Mg/Kg 0	3.75	7.5	15.0	30.0	60.0
Luekocyte Count	Total Count	mm^3 (X ± SE)	6149±288	5788±281	5477±291	6553±271	7087±444	2869±479[a]
	Lymphocyte	mm^3	5083±220	4723±197	4603±245	5400±248	5830±405	2007±489[a]
	Neutrophils	mm^3	980±99	1034±154	834±80	1063±69	1197±99	622±149
	Monocytes	mm^3	65±16	30±10	33±16	76±25	53±22	22±13
Organ Weight	Spleen Real	mg (X ± SE)	496±13	504±9	484±10	454±10[a]	424±9[a]	249±18[a]
	Spleen Body	x 10^{-3}	2.22±.05	2.17±0.04	2.14±.05	2.0±.03[a]	1.97±.04[a]	1.84±.11[a]
	Thymus/Real	mg	314±9	327±10	317±9	308±7	272±10[a]	57±12[a]
	Thymus/Body	x 10^{-3}	1.41±0.04	1.41±0.06	1.4±0.04	1.36±0.02	1.27±0.04	0.42±0.08[a]
Mitogen Response	CON A	(X ± SE) CPM x 10^{-3}	47.5±3.1	51.7±3.6	48.5±3.4	51.4±3.0	41.1±4.7	14.1±12[a]
	PHA	CPM x 10^{-3}	24.4±8.8	23.3±6.9	19.9±3.1	20.8±5.0	12.6±2.9	3.7±3.3
	PWM	CPM x 10^{-3}	33.0±2.4	28.6±1.7	27.8±0.3	29.7±1.2	23.2±3.6[a]	8.7±3.7[a]
	STM	CPM x 10^{-3}	11.7±6	9.7±0.5	11.3±3.1	10.5±1.6	5.6±1.6	3.2±3.2
NK Cell	YAC	(X ± SE) % Release	34.7±1.5	32±1.9	31.5±1.9	31.8±2.3	31.3±1.8	24.2±1.6[a]
	W/FU	50:1	15.0±2.2	15.8±2.3	14.5±1.8	16.1±2.7	15.5±1.8	12.3±4.1

CHLORDECONE		Units	Mg/Kg 0	0.625	1.25	2.5	5.0	10.0
Luekocyte Count	Total Count	mm^3 (X ± SE)	5984±323	6294±231	6754±255	6568±333	6055±169	4768±512[a]
	Lymphocyte	mm^3	5275±243	5564±228	5861±300	5758±263	5444±148	4397±458
	Neutrophils	mm^3	596±119	612±86	780±84	677±146	509±83	290±57
	Monocytes	mm^3	113±12	118±14	113±13	113±21	102±17	81±14
Organ Weight	Spleen Real	mg (X ± SE)	502±10	494±9	510±9	516±8	531±8	380±.19[a]
	Spleen/Body	x 10^{-3}	2.22±.04	2.18±.04	2.23±.03	2.25±.03	2.32±.03	2.06±07[a]
	Thymus Real	mg	272±9	281±14	294±9	301±9	293±13	182±25[a]
	Thymus/Body	x 10^{-3}	1.21±05	1.24±.06	1.28±.04	1.31±.03	1.28±.05	0.97±.13[a]
Mitogen Response	CON A	(X ± SE) CPM x 10^{-3}	56.0±2.8	63.5±4.5	66.3±5.6	60±3.5	60.5±3.4	31.5±2.7[a]
	PHA	CPM x 10^{-3}	38.1±4.9	38.1±4.9	31.4±4.1	21.9±1.2[a]	29.6±3.4[a]	43.1±3.3
	PWM	CPM x 10^{-3}	40.1±3.5	35.9±3.0	35.3±3.7	28.9±2.5[a]	38.3±2.7	43.9±2.1
	STM	CPM x 10^{-3}	19.8±3.0	16.5±1.9	14.5±1.6	11.8±2.0[a]	18.6±2.8	30.4±1.2[a]
NK Cell	YAC	(X ± SE) % Release	16.0±0.8	14.3±1.0	15.0±1.0	15.9±1.0	14.4±.8	6.5±0.7[a]
	W/FU	50:1	6.1±.7	4.6±0.9	5.5±0.6	7.0±0.8	5.5±1.3	2.5±.5[a]

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.

Table 15: RESULTS OF CYCLOPHOSPHAMIDE AND DIETHYLSTILBESTROL IN IMMUNOLOGY WITH THE TOXICOLOGICAL SCREEN

CYCLOPHOSPHAMIDE		UNITS	Mg/Kg 0	1.5	3.0	6.0	12.0	24.0
Luekocyte Count	Total Count	mm^3 (X ± SE)	6424±388	4425±243[a]	2990±135[a]	1733±72[a]	1205±63[a]	128±19[a]
	Lymphocyte	mm^3	5656±323	3744±257[a]	2332±108[a]	1320±73[a]	953±51[a]	6±2[a]
	Neutrophils	mm^3	663±111	525±91	609±84	368±60	228±43[a]	1±0[a]
	Monocytes	mm^3	89±12	65±10	49±8[a]	21±3[a]	19±2[a]	NT[b]
Organ Weight	Spleen Real	mg (X ± SE)	511±12	452±10[a]	390±6[a]	328±7[a]	269±5[a]	215±21[a]
	Spleen/Body	g/kg	2.20±0.04	1.95±0.02[a]	1.7±0.03[a]	1.4±0.03[a]	1.3±0.03[a]	1.3±0.09[a]
	Thymus Real	mg	282±15	244±15	182±7[a]	82.5±5[a]	52±3[a]	5±4[a]
	Thymus/Body	$\times 10^{-3}$	1.2±0.06	105±0.06[a]	0.8±0.03[a]	0.4±0.02[a]	0.2±0.01[a]	0.03±0.02[a]
Mitogen Response	CON A	(X ± SE) CPM $\times 10^{-3}$	71.2±3.7	47±5.8[a]	34.7±7.6[a]	27±1.7[a]	NT	NT
	PHA	CPM $\times 10^{-3}$	56.4±6.7	20.9±2.3[a]	20.9±4.0[a]	18.8±2.2[a]	NT	NT
	PWM	CPM $\times 10^{-3}$	55.5±1.9	43.3±5.3	25.1±6.8[a]	10.3±0.8[a]	NT	NT
	STM	CPM $\times 10^{-3}$	13.9±2.2	3.3±0.6[a]	1.4±0.4[a]	0.8±0.4[a]	NT	NT
NK Cell	YAC	(X ± SE) % Release	19.8±1.5	21±0.9	22.9±1.3	11±1.2[a]	3.2±0.5[a]	NT
	W/FU	50:1	9.5±0.6	9.0±0.6	8.2±0.8	2.2±0.7[a]	1.5±0.5[a]	NT

DIETHYLSTILBESTROL		Units	Mg/Kg 0	31.25	62.5	125	250	500
Luekocyte Count	Total Count	mm^3 (X ± SE)	6513±321	4320±344	5785±350	6520±313	6518±273	11487±3993
	Lymphocyte	mm^3	5318±324	3545±296	4845±252	5092±278	5399±220	8861±3305
	Neutrophils	mm^3	1143±182	725±87	824±120	1363±185	1024±122	2568±733[a]
	Monocytes	mm^3	45±17	43±16	103±33	65±21	84±29	57±17
Organ Weight	Spleen Real	mg (X ± SE)	491±10	317±10[a]	350±10[a]	353±10[a]	367±6[a]	298±24[a]
	Spleen/Body	$\times 10^{-3}$	2.31±0.05	2.19±0.05	2.23±0.04	2.16±0.03	2.14±0.04	2.04±0.1
	Thymus Real	mg	311±9	135±5[a]	143±7[a]	151±9[a]	150±5[a]	67±9[a]
	Thymus/Body	$\times 10^{-3}$	1.46±0.04	0.93±0.2[a]	0.91±0.04[a]	0.92±0.05[a]	0.87±0.03[a]	0.45±0.05[a]
Mitogen Response	CON A	(X ± SE) CPM $\times 10^{-3}$	60.1±5.7	63.4±13.7	75.5±5.5	64.9±7.9	56.8±10	67.8±3.9
	PHA	CPM $\times 10^{-3}$	17.6±3.3	57.4±5.2[a]	45.9±5.9[a]	29.7±3.4	24.9±4.7	44.7±9.1[a]
	PWM	CPM $\times 10^{-3}$	31.6±2.3	44.8±1.4[a]	37.6±3.2	30.3±1.3	27.7±3.8	33.1±2.7
	STM	CPM $\times 10^{-3}$	11.6±1.7	31.7±4.1[a]	28.9±3.9[a]	13.6±2.5	12.7±3.2	19.5±1.9
NK Cell	YAC	(X ± SE) % Release	25.1±2.6	23.2±2.9	21.2±2.1	23.6±1.9	22.8±1.8	21.7±2.9
	W/FU	50:1	11.8±0.9	10.3±1.1	8.9±0.7	10.6±0.9	9.8±1.0	6.6±1.7[a]

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.

E. REPRODUCTIVE TOXICOLOGY/TERATOLOGY

The assessment of reproductive toxicity and teratology required two different experimental protocols since they cover different processes and organ systems. A compound may affect either the male reproductive system (testes and accessory sex glands) or the female reproductive system (ovarian, uterus; and associated structures); the endocrine components responsible for the proper functioning of either system; or the implantation, embryonic and/or fetal processes responsible for development of the egg to the full term fetus. It was decided to concentrate our efforts upon two of these areas - male reproduction and teratology. A series of tests to evaluate key male reproductive parameters as well as a teratology screen were used. The male reproductive parameters included testes weight, sperm concentration, sperm motility, FSH levels, and testicular pathology. The teratology screen was developed at the HERL and follows the progress of neonates exposed in utero through the early postnatal period as an indicator of developmental toxicity. Such tests were evaluated in these and other studies for their sensitivity and accuracy as indicators of the potential toxic effects of xenobiotics. The results are given in Tables 16 to 18.

Acrylamide

Except in the group receiving 60 mg/kg acrylamide, no significant effects were seen in the measured reproductive parameters. Effects at the highest dosage included reduced testes weight, sperm motility, and serum testosterone and increased incidence of testicular histopathology and percent abnormal sperm morphology. These effects are more likely due to the general inatiation of the animal and not to any specific reproductive toxicity. In dams treated with acrylamide for ten days, there was no significant deviation from controls

either in litter size or in the average pup weight up to three days post-partum.

Chlordecone

This compound produced positive results in both the teratology (10 mg/ kg/day) and reproduction parts (0.625 to 10 mg/ kg/day) of the test protocol. Although chlordecone has previously been shown to be non-teratogenic in the rat, other studies have indicated that it does induce functional deficits which do not manifest themselves until the postnatal period[13,17]. Thus, for chlordecone the screen is more sensitive than the normal teratology bioassay. The reproductive toxicology portion of the screen was especially informative since alterations in sperm concentration were seen at all doses tested even at doses where no other adverse effects were noted. This is noteworthy since chlordecone is a human male sterilant and has severely reduced human sperm levels in populations of male workers exposed to the compound in the work-place[41].

Cyclophosphamide

This compound produced no positive effects in the reproductive toxicity that could not be attributed to overt toxicity. There are several reports, however, which identify cyclosphamide as a reproductive toxin[42,43,44,45,46]. Cyclophosphamide is also a well-known teratogen capable of producing a variety of severe malformations[47]. The single dose tested in the neonatal survival screen (12 mg/kg/day) resulted in a complete lack of live births, indicating severe developmental damage.

Diethylstilbestrol

DES was positive in both the reproductive toxicity and teratology portions of the screen. Significant reductions were seen in testes weight and serum testosterone levels in all DES dose groups and in sperm concentration at a dose of 125 mg/kg and higher. Adequate motility evaluations were not possible due to severe semen agglutination in all samples. Significant testicular pathology was noted in all but one animal receiving DES. DES treatment of 15 mg/kg to the dam dam for 10 consecutive days resulted in total *in utero* embryo/fetal death. These results were as expected since this compound is a synthetic estrogen and is a well-known human teratogen, affecting the reproductive system of both male and female children exposed to the chemical during gestation. FSH level determinations were not performed in this study since these and other studies had shown this procedure to be time-consuming and too costly for a general screen. It is recommended, therefore, that this test be dropped from any proposed screen. Similarly, although testicular pathology was a good indicator of toxicity for DES, it is felt that testicular pathology is not sufficiently sensitive to be worth the effort and expense involved in conducting such studies.

Table 16: RESULTS OBTAINED IN REPRODUCTIVE TOXICITY AND TERATOLOGY WITH THE TOX SCREEN

ACRYLAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (20)	Vehicle Control (24)	3.75 (10)	7.5 (10)	15.0 (34)	30.0 (10)	60 (10)
REPRODUCTIVE TOXICOLOGY							
TESTES WEIGHT	-[a]	-	-	-	-	-	+
SPERM CONC.	-	-	-	-	-	-	-
SPERM MOTILITY	-	-	-	-	-	-	+
TESTOSTERONE LEVEL	-	-	-	-	-	-	+
FSH LEVEL	-	-	-	+	-	-	-
TESTICULAR PATHOLOGY	-	-	-	-	-	-	+
TERATOLOGY							
NEONATAL SURVIVAL	-	-	NT[b]	NT	-	NT	NT
SCE: MATERNAL/EMBRYO	-	-	NT	NT	-	NT	NT

CHLORDECONE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (20)	Vehicle Control (24)	0.625 (10)	1.25 (10)	2.5 (10)	5.0 (10)	10.0 (34)
REPRODUCTIVE TOXICOLOGY							
TESTES WEIGHT	-	-	-	-	-	-	+
SPERM CONC.	-	-	+	+	+	+	+
SPERM MOTILITY	-	-	-	-	-	-	-
TESTOSTERONE LEVEL	-	-	-	-	-	-	NT
FSH LEVEL	-	-	-	-	-	-	NT
TESTICULAR PATHOLOGY	-	-	-	-	-	-	-
TERATOLOGY							
NEONATAL SURVIVAL	-	-	NT	NT	NT	NT	+
SCE: MATERNAL/EMBRYO	NT	-	NT	NT	NT	NT	-

CYCLOPHOSPHAMIDE DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (20)	Vehicle Control (24)	1.5 (10)	3.0 (10)	6.0 (10)	12.0 (30)	24.0 (10)
REPRODUCTIVE TOXICOLOGY							
TESTES WEIGHT	-	-	-	-	-	-	+
SPERM CONC.	-	-	-	-	-	-	-
SPERM MOTILITY	-	-	-	-	-	-	-
TESTOSTERONE LEVEL	-	-	-	-	-	-	+
FSH LEVEL	-	-	-	-	-	-	-
TESTICULAR PATHOLOGY	-	-	-	-	-	-	-
TERATOLOGY							
NEONATAL SURVIVAL	-	-	NT	NT	NT	+	NT
SCE: MATERNAL/EMBRYO	NT	-	NT	NT	NT	NT	NT

DES DOSAGE; MG/KG (ANIMALS/GROUP)	Cage Control (20)	Vehicle Control (24)	31.25 (14)	62.50 (14)	125 (14)	250 (10)	500 (10)
REPRODUCTIVE TOXICOLOGY							
TESTES WEIGHT	-	-	+	+	+	+	+
SPERM CONC.	-	-	-	-	+	+	+
SPERM MOTILITY	-	-	-	NT	NT	NT	NT
TESTOSTERONE LEVEL	-	-	+	+	+	+	+
FSH LEVEL	NT	NT	NT	NT	NT	NT	NT
TESTICULAR PATHOLOGY	-	-	+	+	+	+	+
TERATOLOGY							
NEONATAL SURVIVAL	-	-	+	NT	NT	NT	NT
SCE: MATERNAL/EMBRYO	NT	-	NT	NT	NT	NT	NT

[a] -, NOT SIGNIFICANTLY DIFFERENT FROM CONTROLS; +, SIGNIFICANTLY DIFFFERENT FROM CONTROLS.
[b] NT, NOT TESTED DUE TO LETHALITES, INSUFFICIENT SAMPLE TISSUE, OR NOT SCHEDULED TO BE EVALUATED.

Table 17: RESULTS OF ACRYLAMIDE AND CHLORDECONE IN REPRODUCTION AND TERATOLOGY WITH THE TOX SCREEN

ACRYLAMIDE		Units	Mg/Kg 0	3.75	7.5	15.0	30.0	60.0
Organ Weight	Testes	grams (X ± SE)	2.7±0.11	2.79±0.09	2.77±0.08	2.75±0.07	2.67±0.1	2.27±0.16[a]
	Testes/Body	x 10^2	1.18	1.2	1.23	1.21	1.24	2.05[a]
	Sperm Conc.	x 10^6/mg fluid	2.15±0.1	2.3±0.12	2.2±0.1	2.4±0.1	2.5±0.1	2.2±0.1
	Sperm Motil.	% Motile sperm	46±21	52±12	49±20	43±18	54±7	17±6[a]
	Testosterone	ng/ml	2.4±0.9	2.5±0.9	3.3±2.7	1.8±1.6	2.3±1.4	<0.012[a]
	FSH Level	ng/ml	9.7±1.6	9.5±1.7	7.9±1.3	8.5±2.3	8.3±2.2	9.4±1.3
	Testicular Path	#Pos/#Evaluated	0/10	0/10	0/10	0/10	0/10	7/7[a]
Neonatal Survival	No. Live d.1	(X ± SE)	7.2±1.3	NT[b]	NT	7.1±0.9	NT	NT
	d.3		7.2±1.3	NT	NT	7.1±1.0	NT	NT
	Avg. Wgt.d.1	grams	5.3±0.08	NT	NT	5.2±0.05	NT	NT
	d.3	grams	6.5±0.01	NT	NT	6.3±0.13	NT	NT
SCE Analysis	Maternal cells	SCE/cell						
	Embryo cells	SCE/cell (X ± SE)	4.3±0.64	NT	NT	5.8±1.2	NT	NT
			4.0±0.77	NT	NT	3.9±0.76	NT	NT

CHLORDECONE		Units	Mg/Kg 0	0.625	1.25	2.5	5.0	10.0
Organ Weight	Testes	grams (X ± SE)	2.78±0.64	2.76±0.12	2.73±0.08	2.73±0.06	2.7±0.09	2.42±0.19[a]
	Testes/Body	x 10^2	1.20	1.24	1.19	1.19	1.18	1.32[a]
	Sperm Conc.	x 10^6/mg fluid	2.18±0.17	2.06±.32[a]	1.93±3.8[a]	2.27±.49[a]	1.92±.48[a]	2.31±.39[a]
	Sperm Motil.	% Motile sperm	42±10	48±11	43±20	34±20	33±16	32±16
	Testosterone	ng/ml	1.8±1.2	1.7±1.4	20±1.8	3.0±2.7	2.2±1.3	-
	FSH Level	ng/ml	13.5±3.1	13.3±3.4	14.2±3.1	14±2.4	14.2±2.1	-
	Testicular Path	#Pos/#Evaluated (X ± SE)	0/10	1/10	0/10	0/10	0/10	1/10
Neonatal Survival	No. Live d.1	(X ± SE)	6.7±2.5	NT	NT	NT	NT	4.5±2.6
	d.3		6.7±2.5	NT	NT	NT	NT	1.1±1.8[a]
	Avg. Wgt.d.1	grams	5.3±0.4	NT	NT	NT	NT	4.8±0.4[a]
	d.3	grams	6.9±0.7	NT	NT	NT	NT	5.1±1.1[a]
SCE Analysis	Maternal cells	SCE/cell	3.0±1.7	NT	NT	NT	NT	3.3±0.5
	Embryo cells	SCE/cell (X ± SE)	4.0±0.8	NT	NT	NT	NT	4.7±0.86

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.

Table 18: RESULTS OF CYCLOPHOSPHAMIDE AND DIETHYLSTILBESTROL IN REPRODUCTION AND TERATOLOGY IN THE TOX SCREEN

CYCLOPHOSPHAMIDE		Units	Mg/Kg 0	1.5	3.0	6.0	12.0	24.0
Organ Weight	Testes	grams (X ± SE)	2.71±0.08	2.72±0.13	2.69±0.06	2.74±0.05	2.64±0.18	2.45±0.11[a]
	Testes/Body	x 10^2	1.17	1.18	1.18	1.20	1.24	1.45[a]
	Sperm Conc.	x 10^6/mg fluid	2.39±0.37	2.27±0.23	2.12±0.29	2.19±0.25	2.28±0.36	2.26±0.24
	Sperm Motil.	% Motile sperm	57±10	55±9	48±11	58±18	51±17	62±6
	Testosterone	ng/ml	1.78±2	1.8±1.39	1.9±1.9	1.8±1.8	1.8±1.3	0.06±0.06[a]
	FSH Level	ng/ml	9.87±1.77	10.1±1.45	9.65±1.38	10.19±1.95	10.88±1.74	8.64±1.93
	Testicular Path	#Pos/#Evaluated (X ± SE)	2/10	3/10	1/10	2/10	4/10	1/3
Neonatal Survival	No. Live d.1		NT[b]	NT	NT	NT	TLR[c]	NT
	d.3		NT	NT	NT	NT	TLR[c]	NT
	Avg. Wgt.d.1	grams	NT	NT	NT	NT	TLR[c]	NT
	d.3	grams	NT	NT	NT	NT	TLR[c]	
SCE Analysis	Maternal cells	SCE/cell	NT	NT	NT	NT	NT	NT
	Embryo cells	SCE/cell	NT	NT	NT	NT	NT	NT

DIETHYLSTILBESTROL		Units	Mg/Kg 0	31.25	62.5	125	250	500
Organ Weight	Testes	grams (X ± SE)	2.52±0.2	1.69±0.11[a]	1.67±0.1[a]	1.65±0.1[a]	1.7±0.15[a]	1.47±0.12[a]
	Testes/Body	x 10^2	1.18	1.17	1.06[a]	1.01[a]	1.00[a]	1.02[a]
	Sperm Conc.	x 10^6/mg fluid	115±24	110±37	102±17	98±17[a]	96±15[a]	83±42[a]
	Sperm Motil.	% Motile sperm	43±14	61	NT	NT	NT	NT
	Testosterone	ng/ml	2.01±1.36	<0.012[a]	<0.012[a]	<0.012[a]	<0.012[a]	<0.012[a]
	FSH Level	ng/ml	NT	NT	NT	NT	NT	NT
	Testicular Path	#Pos/#Evaluated (X ± SE)	0/10	10/10[a]	10/10[a]	10/10[a]	9/10[a]	10/10[a]
Neonatal Survival	No. Live d.1	(X ± SE)	7.4±1.9	TLR[c,d]	NT	NT	NT	NT
	d.3		7.8±0.9	TLR[c,d]	NT	NT	NT	NT
	Avg. Wgt.d.1	grams	5.4±0.25	TLR[c,d]	NT	NT	NT	NT
	d.3	grams	6.8±1.05	TLR[c,d]	NT	NT	NT	NT
SCE Analysis	Maternal cells	SCE/cell	3.6±0.7	NT	-	-	-	-
	Embryo cells	SCE/cell (X ± SE)	3.1±0.53	NT	-	-	-	-

[a]DENOTES A STATISTICALLY SIGNIFICANT RESPONSE WHEN COMPARED TO VEHICLE CONTROLS.
[b]NT, NOT TESTED DUE TO INSUFFICIENT SAMPLE SIZE, LOSS OF SAMPLE OR NOT SCHEDULED TO BE EVALUATED.

SECTION 6

COMPARISON OF TOXICOLOGICAL SCREEN RESULTS TO THE LITERATURE

The development of a new biological test protocol requires several steps in its validation to establish sensitivity, specificity and accuracy. One crucial step in the validation is to compare the results obtained experimentally with the pure control compounds to data in the literature. The adequacy of the test method to detect the specific toxicological effects associated with the compounds is a necessary part of the validation.

The results of the four toxic chemicals in the Tox Screen compared to the data in the literature on these compounds is given in summary form in Table 19. A discussion with respect to each chemical is given below.

ACRYLAMIDE

Acrylamide neurotoxicity has been described in both man and animals[48]. In humans with prolonged exposure to acrylamide, signs and symptoms include: numbness, muscle weakness, loss of body wieght, apathy, loss of proprioceptive sensations and various motor deficits such as ataxia, foot-drop and muscle atrophy[49]. Results in this study indicate that the figure-8 maze provided a sensitive measure of neurotoxicity and resulted in a dose response effect with exposure of animals to the toxin. Reliable decreases were seen in adult rats following a cummulative dose of 150-600 mg/kg. These data also suggest that both behavioral measures (figure-8 maze activity and acoustic startle response) using a 10-day exposure regime reliably predicted the neurotoxicity associated with chronic exposure to acrylamide. In addition, changes in body and real and relative organ weights and the bladder distention observed in

the animals is also consistent with the literature and with the suspected neural paralytic toxicity of acrylamide[50]. For mutagenesis, immunotoxicology, reproductive toxicology and teratology there is no literature data available for acrylamide.

TABLE 19: RESULTS OF FOUR TOXIC CHEMICALS IN THE TOXICOLOGICAL SCREEN COMPARED TO LITERATURE DATA

	ACRLYAMIDE		CHLORDECONE		CYCLOPHOSP		DES	
	SCREEN	DATA	SCREEN	DATA	SCREEN	DATA	SCREEN	DATA
GENERAL TOX	+[a]	+	+	+	+	+	+	+
MUTAGENESIS	+	NA[b]	-	NA	+	+	-	-
NEUROTOX	+	+	+	+	-	NA	-	NA
IMMUNOTOX	-	NA	+	NA	+	+	+[c]	+
REPROTOX	-	NA	+	+	+[c]	+	+	+
TERATOLOGY	-	NA	+	+	+	+	+	+

[a]A positive response for both the screen results and literature data is defined as evidence of a specific toxicological effect supported by dose response data.
[b]NA, not available.
[c]Positive response resulting from a generalized toxicological response (see text for specific toxin and discipline).

CHLORDECONE

Chlordecone is a chlorinated hydrocarbon insectide whose primary targets are the nervous and reproductive systems. Prominent signs of poisoning in both humans and laboratory animals are tremors, hyperexcitability, male sterility and functional deficits in the embryo/fetus[41,51]. The increased amplitude of the acoustic startle response of animals exposed to chlordecone in the present experiment is consistent with previous research[26,27,28]. Motor activity in figure-8 mazes had been previously reported to increase following three week dietary exposure to 40-80 ppm chlordecone[26]. This hyperactivity was not replicated in the present experiment, although several factors including the route of administration (dietary vs intubation) and strain of rat (Sprague Dawley vs Fischer 344) could account for this discrepancy. More recently, Pryor et al.[28] reported that oral administration of chlordecone to Fischer 344 rats did not affect motor activity in the open field. This finding, in combination with data from the present experiment, argues that Fischer 344 rats may not exhibit hyperactivity as a consequence of chlordecone exposure. In addition to neurotoxic effects, chlordecone caused a reduction in sperm concentration at all test concentrations and a positive effect on neonatal survival. These observations confirm acrylamide as a reproductive toxin. Unfortunately, no data are available for the immunotoxic and mutagenic effects of acrylamide.

CYCLOPHOSPHAMIDE

Cyclophosphamide is an agent which is cytotoxic due to its cross-linking capabilities with DNA and other essential macromolecules[52]. The results obtained with this compound and SCE analysis are consistent with numerous other studies indicating cyclophosphamide has potent SCE-inducing activity

after host activation[24,25]. Phosphoramide mustard is considered to be one of the more potent cytotoxic metabolites of cyclophosphamide and is detectable in the urine of treated rats[53,54]. Although the specific metabolite(s) of cyclophosphamide treated rats was not identified as part of this program, the mutagenic potential of the urine was confirmed using the Ames test. The results of this study also verify that cyclophosphamide is a very potent immunosuppressant and are in agreement with reports which show cyclophosphamide to significantly suppress the mitogen response of rat pheripheral blood lymphocytes to Con A, to affect B-lymphocyte function, and to affect NK activity[31,32,34,35]. Cyclophosphamide is also known to affect uridine and thymidine uptake by spermatogonia, to affect the morphology of spermatocytes, spermatids, and spermatozoa, and to produce a series of severe malformations in the embryo/fetus. The reproductive evaluations reported in this study show cyclophosphamide to be a reproductive toxin using testes weight, serum testosterone concentration reduction and neonatal survival as the cirteria. A number of general toxic effects, such as reduction in body and organ weights and alterations in serum chemistry are also expected with cyclophosphamide. Although there are no reports in the literature demonstrating the neurotoxic effects of cyclophosphamide, it would be expected to have little affect on the mature nervous system[29]. This is due to the fact that neurogenesis is completed in the early postnatal period in both humans and rats and the mature nervous system would not likely be affected by the type of chemical toxicity cyclophosphamide is capable of producing.

DIETHYLSTILBESTROL

Diethylstilbestrol (DES) is a nonsteriod synthetic estrogen which was prescribed for complications of pregnancy between 1940 and the early 1970's[55].

In utero exposure in humans has been shown to produce abnormalities of the reproductive tract[55]. In the rat, the central nervous system is highly sensitive to estrogens during the first few days of postnatal life. During this period a single treatment with a natural or synthetic estrogen can permanently alter the development of the brain, producing effects which are manifest in later life. Exposure of DES to adult rats in the study produced deficits in both motor activity and sensorimotor responding. The effects observed here are partially in agreement with those observed for two chlorinated hyrocarbon pesticides with estrogenic properities (DDT and chlordecone)[56,57]. These compounds resulted in increased acoustic startle response and decreased motor activity. However, these compounds also produced a general increased excitability, and at high dosages, tremors which would result in increased acoustic startle reactivity. Unfortunately, there are no reports on the neurotoxic effects of DES. DES has been reported to affect the immune responsiveness of female mice causing suppressions in the lymphoproliferative response to Con A, PHA, and LPS in the adult mouse and reductions in NK activity of perinatally treated adults[38]. This study shows enhancements in the lymphoproliferative response to PHA, PWM and STM; no significant depression in responsiveness was seen at any dose level. A suppression in NK activity was also observed. However, this is due to a spuriously low response by one animal in the dosage group. It appears that DES does not adversely affect the immune system of adult male rats and that its specific effects are on the immune system depending on the age, sex, and species employed.

The dosages of DES utilized for this study caused severe inanition and were probably an order of magnitude too high than what is required to produce the same responses. Most measurements resulting in positive responses in all

areas of toxicity were due to extreme generalized toxicity and not specific toxic effects. The positive reproductive and neonatal survival effects seen in this study with DES are consistent with the literature.

SECTION 7

DISCUSSION

A. VALIDATION OF THE TOXICOLOGICAL SCREEN

Before the Tox Screen can be utilized with complex waste samples, its ability to detect biologically active pure compounds and reference material complex waste mixtures must be determined. Although each test in the Tox Screen has been validated independently, it is necessary to validate the Tox Screen and its components as a single integrated protocol. An initial validation was performed with a number of positive control toxins of known and defined toxicity.

There were basically three goals in performing a validation study. The first goal of the validation study was to determine if the Tox Screen could detect the biological activity associated with the pure compounds. This goal could be described more specifically as determining the sensitivity, specificity, and accuracy of the Tox Screen. (These statistical parameters will be described fully in the section on statistical considerations). The second goal of the Tox Screen was to determine whether the screen could detect the type of toxicological activity associated with the pure compounds as given in the literature. The results obtained with compounds of known chronic effects in this screen would allow for the evaluation of the screen's ability as a predictive tool for identifying the type of chronic effects which may be assoaciated with an unknown waste sample. The third goal of the validation study was to identify which biological endpoints employed in the screen were most predictive of the toxic potential of compounds. That is, the goal was to identify those tests which were the most sensitive, specific,

and hence, the most accurate. The identification of the most accurate test played an important role in the validation study. If after the evaluation of a number of compounds such tests did become apparent, the program design called for the utilization of those sensitive assays in a mini screen, or what has been entitled the Prescreen, for use in prioritizing waste samples to be evaluated in the full Tox Screen. Also, those tests which proved to be less useful in the full Tox Screen could be deleted and replaced with more meaningful tests.

An example of a hypothetical prescreen protocol is given in Figure 4. The treatment protocol for the prescreen is an abbreviated version of that utilized in the Tox Screen. In the prescreen the treatment protocol involves the exposure of 10 male animals by gavage to one concentration of the waste ($\leq$ 1g/kg) for 10 consecutive days. It is believed that this single large dosage for 10 days will facilitate the identification or ranking of biologically active wastes. This selection of a single dosage level is not a result of the validation study with a number of compounds in the full Tox Screen. It is an a priori decision made by members of the research team to help expedite the screening process and to help develop a simple, rapid and cost effective test for use by OSW.

As mentioned earlier, however, the best or most sensitive tests for use in the prescreen would be apparent only after the results of an extensive validation study have been examined. The hypothetical prescreen in Figure 4 assumes that changes in body weight and death, the Ames test with the pure wastes, the neurotoxicological assessments of the Figure 8 maze and the acoustic startle response were identified as the most sensitive by the validation study results. Due to the large number of RCRA samples requiring evaluation it is believed that the prescreen with its abbreviated treatment protocol and

Hypothetical Prescreen Protocol

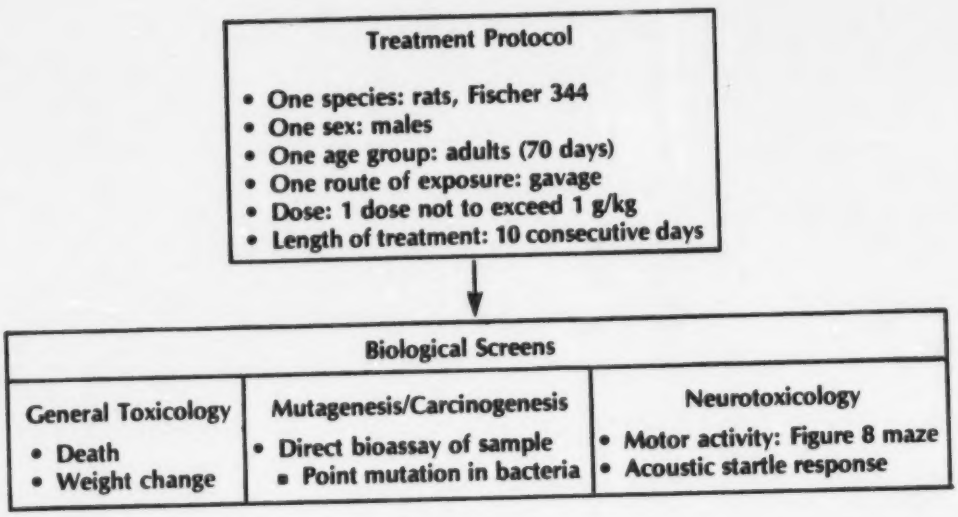

Treatment Protocol
• One species: rats, Fischer 344
• One sex: males
• One age group: adults (70 days)
• One route of exposure: gavage
• Dose: 1 dose not to exceed 1 g/kg
• Length of treatment: 10 consecutive days
Biological Screens
General Toxicology
• Death
• Weight change
Mutagenesis/Carcinogenesis
• Direct bioassay of sample
▪ Point mutation in bacteria
Neurotoxicology
• Motor activity: Figure 8 maze
• Acoustic startle response

Figure 4

its limited number of *in vitro*/*in vivo* tests would serve to facilitate in identifying and prioritizing biologically active samples for evaluation in the full Tox Screen.

Caution must be employed when reviewing results obtained from waste evaluation in the prescreen. Any waste sample which is not active in the prescreen would not be considered a "safe" sample; it would remain a candidate for the Tox Screen but at a lower priority level. And conversely, any sample demonstrating activity or toxicity in the prescreen would not be re-evaluated at a lower concentration in the prescreen but would become an immediate candidate for the Tox Screen.

The prescreen concept was conceived for the single purpose of prioritizing compounds for toxicological testing. It can not be used for other purposes for reasons described below. Because the treatment protocol uses only one exposure concentration, no dose response data will be generated with the prescreen. The use of dose response data is an integral step in defining a chemical's true toxicological potential. Secondly, the prescreen is likely to result in a limited degree of chemical characterization capability due to the reduced number of toxicological *in vitro*/*in vivo* assays it may employ. For these reasons it is strongly felt by the research team and the peer review panel that the information obtained from the prescreen per se cannot aid in regulatory or disposal decision making processes but must be limited to the prioritization process.

B. STATISTICAL CONSIDERATIONS FOR THE TOXICOLOGICAL SCREEN

In an effort to select the "best" tests for use in the prescreen, the sensitivity, specificity, and accuracy of each test in the Tox Screen must first be defined. Sensitivity is the screen's ability to identify a

"positive" toxin as positive, specificity is its ability to identify a "negative" toxin as negative, and accuracy is the screen's ability to identify a combination of positive and negative toxins correctly. These statistical parameters are described mathematically in Figure 5. Once these parameters are established for each test, then the tests can be ranked with respect to their performace in accuracy, and the "best" tests can be selected for the prescreen while the less effective tests are considered for deletion from the Tox Screen.

The current program design requires that the Tox Screen have a sensitivity of 90% and a specificity of 75%. These values were suggested by OSW as their minimum requirements for test accuracy. In order to achieve these levels a large number of compounds must be evaluated in the screen's validation study. The calculation of the number of compounds required is described below.

In order to statistically evaluate each biological screen as a candidate for the prescreen test and to determine the overall accuracy of the Tox Screen, the sensitivity of each test must first be evaluated. If a test is truly 90% sensitive then the range of sensitivities one would expect to see, when challenging that test with 15 to 20 compounds, would be 75% to 99%. This range is a 90% confidence interval based on a binomial distribution. If as few as 10 compounds were selected to evaluate the test, then the expected range of scores would be anywhere from 65% to 99%. This minimum sensitivity or lower 90% confidence limit of 65% obtained with the evaluation of 10 compounds is much too close to chance (50%) to use as one's criteria for sensitivity. Therefore, it is clear that a minimum of 15 to 20 compounds are required to evaluate the sensitivity of each test and to insure a minimum sensitivity of 75% for each test. The fewer the number of compounds evaluated,

Definition of Scores Useful in Prescreen Test Selection

		Control Compound +	Control Compound −	
Test Result	+	a	b	a + b
	−	c	d	c + d
		a + c	b + d	N

Several useful scores for each test may be defined as follows:

Sensitivity = a/(a + c)
Specificity = d/(b + d)
Accuracy = (a + d)/N

Figure 5

the larger is the variance around the true sensitivity of the test. Tighter confidence limits require a greater number of compounds. Assuming that the 15-20 positive toxins could be used for each of the 6 disciplines in the the Tox Screen, then a total of 90 to 120 compounds would be required to confirm the sensitivity of the Tox Screen. This number, however, assumes no overlap in the toxicity of the compounds. If compounds were selected that possess 2 or 3 types of toxicity (i.e., a compound which is an immunotoxin as well as a genetic toxin and possibly a neurotoxin), then the number of compounds required for validation could be reduced.

At 75% specificity the 90% confidence interval ranges from 50% to 99%. Once again this is based on the evaluation of 15 to 20 compounds in the test and assures a minimum specificity of only 50%. The specificity level for the Tox Screen is lower than is the sensitivity level because the acceptable level of false positives (> 50%) is more acceptable than is the generation of false negatives (> 75%) when evaluating waste samples. In additon, the use of a total of just 15 to 20 negative control compounds assumes that 20 compounds could be identified as negative in all tests.

The total number of compounds required to confirm 90% sensitivity and 75% specificity for the Tox Screen is 105 to 140 with the assumptions outlined above. If the sensitivity and specificity levels were changed, then the number of compounds required to complete validation of the Tox Screen would change accordingly. Such changes are under consideration. However, if changes are to be made it is important that the statistical parameters are identified a priori based on subjective considerations by OSW and the research team.

C. RESOLUTION AND FUTURE PLANS

The experimental results presented in this report establish the Toxicological Screen's potential as a useful screening tool. The mini-validation study aided in determining the feasibility of the test protocol and helped to identify potential problems. It was fully understood that 4 compounds were too few to define the sensitivity, specificity, and accuracy of the Tox Screen and to select the "best" tests. As a result several key issues concerning the screen's design and validation must be addressed before it can be applied with complex mixtures.

With respect to the validation of the Tox Screen, the Office of Solid Waste is reconsidering the required 90% sensitivity and 75% specificity levels. In addition, since each assay employed in the Tox Screen has been validated independently, there may not be the need to persue so stringent a validation process. This is supported by the fact that the Tox Screen will not be used by itself for the listing process. Information derived from the evaluation of wastes in the screen will be utilized along with the traditional methods employed by OSW for hazard assessment.

The current research strategy is to modify the screen in two areas. The first area requires short-term research for the selection (addition and elimination) of a few assays within each discipline of the screen to more accurately represent the range of toxicological effects which might be expected from hazardous waste samples. This involves the developement of several short-term bioassays in genetic toxicology, neurotoxicology, immunotoxicology, target organ toxicology, and development. This first area will also involve a review of the cost effectivness of each assay. The second area addresses some basic scientific issues pertinent to the screen's

success and risk assessment capabilities: acute vs chronic exposure and the extrapolation of *in vivo* to *in vitro* results. This process will require long-term research to answer some key issues in toxicological assessment.

The current goal is to utilize information obtained from the short-term research in combination with our current knowledge and the validation study results to suggest *a priori* a prescreen for use by OSW. Once defined, the prescreen can be immediately challenged with several hazardous waste samples and its performance can be evaluated. This modification of the screen, however, will be an ongoing process as additional long- and short-term research results become available and can be incorporated into the screen.

The following paragraphs outline the long-term and short-term research projects for the Hazardous Waste Program.

The Tox Screen is being re-evaluated in order to improve the overall performance of the assay. Suggestions for the addition of relevent bioassays and the elimination of those bioassays which proved not to be useful are given below.

With respect to general toxicity, the broad battery of serum chemistry measurements will be eliminated except for those assessing liver function. All urinalysis measurements, except for pH, will be eliminated. In genetic toxicology SCE's will not be assessed due to cost and time considerations. The immunotoxicity measurements of mitogen response and the natural killer cell assay as well as the reproduction/teratology measurements of FSH levels, testosterone levels, and testicular pathology will be eliminated due to their complexity and lack of cost effectiveness.

The development and addition of several new bioassays to the Tox Screen is in progress. In genetic toxicology sentinel test systems are being developed to act as early warning indicators at waste disposal sites. Other

genetic assays that would be complementary to the currently employed Ames test are the SOS response test in bacteria, phange induction in E. coli, and a human cell mutagenesis system using a DNA shuttle vector technique and DNA sequencing. For rapidly assessing neurotoxic potential, a functional observational battery in rodents is being validated with environmentally relevant chemicals of known and defined toxicity. These results will be compared with more traditional evaluations of nervous system integrity. Once validated, this system will be applied with complex waste samples. A number of tests were employed in the Tox Screen to evaluate immunotoxicity. This results from the fact that this science is still developing short-term, efficient assays. For the Hazardous Waste Program, research will be conducted to identify those cost- and time-effective immunotoxicology assays and endpoints which most readily and reliably identify immunotoxicants. Once identified these assays will be employed in the Tox Screen.

Two new areas of research are being pursued in developmental toxicology. The first is the development of a short-term assay for the detection of spontaneous abortions in rodents following exposure to hazardous wastes and pure chemicals. The second is to demonstrate the applicability of in vitro developmental assays to ecomomically, efficiently and rapidly identify biologically active hazardous waste samples.

To assess the adequacy of a 10-day exposure to predict chronic effects, a research project examining the reversibility and latency of various toxicological effects following 1, 5, and 10 days of exposure has been initiated. The toxicological endpoints to be investigated as part of this study are in the areas of general toxicology, neurotoxicology, and liver toxicity. As part of this effort various modes of exposure will be evaluated. Where feasible, the effects observed in these short-term studies will be compared

to sub-chronic (90-day) exposures. The final results of this study will add to our knowledge of acute to chronic extrapolations as well as aid in the modification of the treatment protocol of the screen with respect to length and mode of exposure.

A second major area of research that is required to support the Tox Screen is the development of extrapolation methods from *in vitro* to *in vivo* experimental systems. Efforts will be made to compare responses in both systems using a series of comparative methods: 1. the use of identical compounds in both *in vivo* and *in vitro* systems 2. the comparison of dose-response data for both systems and 3. the comparison of *in vitro* organ cell systems to whole aninal *in vivo* organ assessments. The development of extrapolation models, especially in a quantative or semi-quantative manner, will permit the use of currently available data bases to support Tox Screen results and the listing process for industrial wastes.

It is evident that the Tox Screen, although currently capable of detecting biological activity, would greatly benefit from the research projects and assay modifications outlined above. The results of the short-term research projects insure the development and incorpoation of assays with pertinent toxicological endpoints. The results also insure the Tox Screen's capability of detecting a wider spectrum of biological activities than it is currently capable of detecting. As data become available on the long term research problems (i.e. acute vs chronic, additivity/synergism/antagonism, *in vitro* vs *in vivo* extrapolation), this information can be used to refine the overall efficiency of the screen, thus increasing its risk assessment value.

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